VAT, the *Thermoplasma* Homolog of Mammalian p97/VCP, Is an N Domain-regulated Protein Unfoldase*§¶

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**The *Thermoplasma* VCP-like ATPase from *Thermoplasma acidophilum* (VAT) ATPase is a member of the two-domain AAA ATPases and homologous to the mammalian p97/VCP and NSF proteins.** We show here that the VAT ATPase complex unfolds green fluorescent protein (GFP) labeled with the ssrA-degradation tag. Increasing the Mg2+ concentration derepresses the ATPase activity and concomitantly stimulates the unfolding activity of VAT. Similarly, the VATAN complex, a mutant of VAT deleted for the N domain, displays up to 24-fold enhanced ATP hydrolysis and 250-fold enhanced GFP unfolding activity when compared with wild-type VAT. To determine the individual contribution of the two AAA domains to ATP hydrolysis and GFP unfolding we performed extensive site-directed mutagenesis of the Walker A, Walker B, sensor-1, and pore residues in both AAA domains. Analysis of the VAT mutant proteins, where ATP hydrolysis was confined to a single AAA domain, revealed that the first domain (D1) is sufficient to exert GFP unfolding indistinguishable from wild-type VAT, while the second AAA domain (D2), although active, is significantly less efficient than wild-type VAT. A single conserved aromatic residue in the D1 section of the pore was found to be essential for GFP unfolding. In contrast, two neighboring residues in the D2 section of the pore had to be exchanged simultaneously, to achieve a drastic inhibition of GFP unfolding.

Proteins of the AAA + superfamily are ubiquitously distributed and involved in an ample variety of cellular processes, for review see a special issue of the Journal of Structural Biology on AAA + proteins (1). A common feature is their assembly into oligomeric complexes, which function in energy-dependent remodeling of proteins or nucleic acids (2). AAA proteins form a subfamily of the AAA + superfamily and group according to the number of AAA domains into type I (one AAA domain) and type II (two AAA domains) proteins. The eukaryotic p97/ VCP proteins belong to the latter family and thus are built of two adjacent AAA domains and were originally suggested to function in membrane fusion (3). Recently, however, it became evident that p97/VCP is primarily involved in extraction and degradation of membrane proteins (4–7), as well as in protein remodeling, e.g. transcription factor mobilization and spindle disassembly (8–11). The cellular importance of p97/VCP function is highlighted by the fact that mutations in p97/VCP were found to cause an ultimately lethal disease, called inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (12). The eukaryotic NSF protein belongs also to the type II AAA family and is essential for vesicular transport and membrane fusion through its interaction with the SNARE proteins (13).

The *Thermoplasma* type II AAA protein VAT was shown before to bind denatured protein substrates and mediate refolding at low Mg2+ concentrations (14). In addition, these experiments suggested, although indirectly, that VAT has an unfolding activity at high Mg2+ concentrations. To study VAT-mediated unfolding in more detail, we employed GFP and GFP11, a fusion of the 11-residue peptide ssrA to the C terminus of GFP (15), as substrates in conjunction with VAT and its deletion variant VATAN. The latter lacks the structurally conserved N domain, suggested earlier to be the substrate-binding domain (16), but still assembles into a hexameric complex (17).

The N domains of VAT, p97, and NSF are structurally conserved and in case of the latter two mediate the interaction with adaptor proteins; adaptor proteins of VAT are not known. The cofactor p47 inhibits the ATPase activity of p97/VCP through its binding to the N domain (18). Similarly, it was shown that binding of α-SNAP to the N domain of the related eukaryotic NSF protein stimulates the ATPase activity (19). The NSF-α-SNAP complex mediates membrane fusion through disassembly of SNARE complexes (13).

Electron tomography and cryo-electron microscopy of single particles revealed a central pore in the hexameric *Thermoplasma* VAT complex (17, 20). Processing of substrates by AAA + proteins was proposed to be achieved by threading through the central pore, which is lined with hydrophobic residues (21). This was recently confirmed by electron microscopy and mutational analysis for *Escherichia coli* proteins of the Clp family and FtsH, as well as for yeast Hsp104 (22–29). However, no unfolding or threading activity has been described for p97/VCP, although it has been shown to suppress polyglutamine-induced protein aggregation in analogy to ClpB (30, 31). In contrast, based on the crystal structure of mouse p97 it was suggested that threading through the pore is not possible, since the channel in D1 was blocked by a zinc ion (32, 33). Experiments with p97/VCP revealed that the main ATP hydrolysis is carried out by its D2 domain (34, 35). In contrast the NSF-D2 domain is catalytically inactive and functions only in hexamerization of the protein, whereby the D1 domain exerts nucleotide hydrolysis (13). NSF was...
shown to disassemble SNARE complexes to recycle the monomers, but protein threading or unfolding has not been demonstrated (13).

In this study we show that the aromatic residues in the D1 and D2 section of the central pore in VAT are involved in unfolding of GFP11. Additionally, we present the mutational analysis of residues in both domains of VAT necessary for nucleotide binding and hydrolysis, i.e. Walker A, Walker B, and sensor-1.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—To generate the mutant variants of VAT, codons for Lys237 and/or Lys542 (Walker A), Glu291 and/or Glu568 (Walker B), Asn334 and/or Asn542 (sensor-1), Tyr264 and/or Trp541, and/or Val542 (pore residues) in plasmid pET-28-VAT-His6 containing the codon optimized vat gene (14) were changed to encode alanine. The plasmid pET-28-VAT-His6 was used to obtain VATwt, a VAT variant deleted for the N-terminal 183 residues, similarly as described for the non-optimised vat gene (17).

**Purification of Proteins**—After transformation of BL21 (DE3) RIL cells with pET-28 constructs of VAT, its mutants, or VATΔN, recombinant clones were grown in LB medium containing 25 mg/liter kanamycin and 34 mg/liter chloramphenicol until an optical density (600 nm) of 1 was reached. Protein production was induced by adding isopropyl 1-thio-β-d-galactopyranoside to a final concentration of 1 mM and the cells were harvested after 4 h and frozen at −20 °C. For the purification of VAT and its mutants (except K237/514A), thawed cells were suspended in 20 mM HEPES, pH 7.5, lysed using a cell disrupter (Avestin) and applied to centrifugation (1 h, 100,000 × g, 25 °C). 3 M NaCl was added to the supernatant to a final concentration of 400 mM, and VAT and its mutants, except for K237/514A, precipitate reversibly in the cold at high salt. The solution was incubated for 3 h at 4 °C, and the precipitated VAT protein was harvested by centrifugation. The protein was resuspended in 20 mM HEPES at 60 °C and precipitated again as described above. This procedure was repeated in total three times. The final protein solution was purified on a Superose-6 column (Amersham Biosciences) equilibrated with 20 mM HEPES, pH 7.5, 150 mM NaCl. Thawed cells containing VAT-K237/514A were suspended in 20 mM HEPES, pH 7.5, 300 mM NaCl, and 10 mM imidazole and processed as described above. The supernatant was applied to nickel-nitrilotriacetic acid beads (Qiagen), and the protein was eluted using a continuous gradient from 20–500 mM imidazole in the suspension buffer. The eluent was purified on a Superose-6 column (Amersham Biosciences) equilibrated with 20 mM HEPES, pH 7.5, 150 mM NaCl. VATΔN cells were suspended in 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, and processed as described above. The supernatant was incubated at 60 °C for 22 min and centrifuged (20 min, 20,000 × g, 4 °C) to remove precipitated *E. coli* proteins. In the following the protein solution was diluted 1:3 in 20 mM HEPES, pH 7.5, and applied to a HiTrap Q column (Amersham Biosciences). The protein was eluted by a discontinuous gradient of 50–400 mM NaCl in 20 mM HEPES, pH 7.5, and 5 mM MgCl2. The fractions containing the VATΔN protein were pooled, loaded onto nickel-nitrilotriacetic acid beads (Qiagen), washed with 20 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM MgCl2, 20 mM imidazole, and eluted with 500 mM imidazole in the same buffer. The eluent was applied to a Superose-6 column (Amersham Biosciences) equilibrated with 20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl2. All mutant VAT proteins were shown to assemble in an ATP-independent manner into hexameric complexes indistinguishable from wild-type VAT complexes, as analyzed by size exclusion chromatography, native gel electrophoresis, and electron microscopy. Wild-type proteasomes, Δα(2–12) 20 S proteasomes, and GFP/GFP11 were purified as described (15, 36).

**Measurement of the ATPase Activity**—The ATPase activity of VAT and VATΔN was determined by the malachite green assay (37). The total reaction volume was 50 μl of HM (50 mM HEPES, pH 7.5, 100 mM NaCl, 120 mM MgCl2, 1 mM dithiothreitol) or LM (50 mM HEPES, pH 7.5, 100 mM NaCl, 20 mM MgCl2, 1 mM dithiothreitol) buffers, as used, indicated. The reaction was stopped by addition of 800 μl of malachite green solution followed by 100 μl of citric acid (34%(w/v)). After 30-min incubation at room temperature, absorption at 640 nm was determined in a UV-visible spectrophotometer (Lambda 40, PerkinElmer Life Sciences). For kinetic analysis the reactions were carried out at different ATP concentrations (60 μM to 3.5 mM), and the data points were fitted according to Michaelis-Menten kinetics using Origin 5.0.

**Fluorescence Assays of GFP/GFP11 Unfolding**—GFP/GFP11 fluorescence was monitored continuously in a fluorescence plate reader (Fluostar Optima, BMG Technologies) using filters of 480 nm for excitation and 520 nm for emission. GFP or GFP11 were diluted, as indicated, and the reactions were started by addition of ATP. The total reaction volume was 200 μl. For the kinetic reactions different amounts of GFP11 from 0 to 2 μM in 0.25 μM steps were incubated in HM or LM buffer, including 50 mM VAT or VATΔN and a 28 mM concentration of Δα-(2–12) 20 S proteasomes. After starting the reactions with 5 μM ATP the initial slopes were recorded and the values fitted according to Michaelis-Menten kinetics using Origin 5.0. The curves were corrected by a control reaction without ATP.

**Western Blot Analysis**—The Western blot analysis of GFP11 degradation by proteasomes was performed with anti-enhanced cyan fluorescent protein (a mutant of GFP) antibodies.

**RESULTS**

**VAT Shows Mg2+-stimulated Unfolding of GFP11**—In search for an assay to demonstrate unfolding activity of VAT directly, we employed GFP11, a C-terminal fusion of the globular green fluorescent protein with the 11-residue peptide ssrA (15). In eubacteria, the ssrA tag is added to the C terminus of incompletely translated proteins stalled at the ribosome, thus marking them for degradation (38). The tagged protein gives the same fluorescence quantum yield and emission spectrum as non-tagged GFP, and its unfolding can be directly observed by loss of fluorescence (15). An ssrA-tagging system has not been identified in archaea, but we have shown before that the archaeal PAN ATPase unfolds GFP11 (39). We measured the unfolding activity of VAT in the presence of 20 mM Mg2+ at 60 °C, the optimal growth temperature of *Thermoplasma*. Since the unfolding activity was extremely low we searched for a stimulating factor. In a previous study it was shown that elevated Mg2+ concentrations stimulated the ATPase activity of VAT (14). Mg2+ induces more flexibility to the rigid structure of VAT and may therefore enhance ATP hydrolysis (see supplemental Fig. S1). As monitored by CD spectroscopy Mg2+ does, however, not influence secondary structure elements of VAT (data not shown). We found here that increasing the Mg2+ concentration to 120 mM stimulated GFP11 unfolding and turned VAT into an unfoldase (Fig. 1A). The assays were carried out in the presence of the *Thermoplasma* Δα-(2–12) 20 S proteasome, which is lacking residues 2–12 of the α subunits. This deletion opens the entrance channel into the proteasome and results in enhanced protein degradation activity (39). The mutant proteasomes degraded unfolded GFP11 (Fig. 1A) and thus prevented its competition with the folded GFP11 in binding to VAT, as can be noticed in a slower unfolding reaction in the absence of proteasomes (see supplemental
The unfolding of GFP11 occurred only in the presence of a hydrolyzable nucleotide, like ATP or CTP and, albeit with lower efficiency, in the presence of GTP or UTP. Unfolding was not detected during incubation with ADP nor the non-hydrolyzable analogues AMP-PNP, AMP-PCP, or ATPγS. Subsequent addition of ADP or ATPγS to a GFP11 unfolding reaction started with ATP caused an immediate inhibition of unfolding (data not shown). Neither an elevated temperature of 60 °C nor an increased Mg<sup>2+</sup> concentration of 120 mM did induce spontaneous unfolding of GFP11 (data not shown). Thus, unfolding of the stable globular protein GFP11 by VAT is dependent on nucleotide hydrolysis.

**VATΔN Is a More Efficient Unfoldase than VAT—**Next, we tested VATΔN, a mutant of VAT deleted for the putative substrate-binding N domain (16, 17), for GFP11 unfolding activity. Unexpectedly, VATΔN showed strongly enhanced unfolding of GFP11 in comparison with wild-type VAT. Moreover, VATΔN displayed this strong unfolding activity at a Mg<sup>2+</sup> concentration as low as 20 mM and was not stimulated further by increasing it to 120 mM (Fig. 1A).

**Mg<sup>2+</sup> Increases the ATP Hydrolysis of VAT but Not of VATΔN—**To clarify the differences in unfolding of GFP11 between VAT and VATΔN, we performed kinetic studies of the ATP hydrolyzing activity of both proteins at Mg<sup>2+</sup> concentrations of 20 and 120 mM, respectively (Fig. 1B). To this end, we measured the release of inorganic phosphate of both proteins at different ATP concentrations and fitted the data points according to Michaelis-Menten kinetics. Additionally, we analyzed the kinetics of GFP11 unfolding in the same way, using different GFP11 concentrations (TABLE ONE). As described above for the GFP11 unfolding activity, VAT showed strongly enhanced ATP hydrolysis at high Mg<sup>2+</sup> conditions. In contrast, the ATPase activity of VATΔN was optimal at low Mg<sup>2+</sup> and slightly inhibited at high Mg<sup>2+</sup>. Importantly, the turnover (k<sub>cat</sub>) of ATP by VATΔN at low Mg<sup>2+</sup> was almost 2- or 20-fold higher as compared with the activity of VAT at high Mg<sup>2+</sup> or low Mg<sup>2+</sup>, respectively (Fig. 1B). Moreover, VAT showed a lower apparent affinity for the nucleotide than VATΔN at both conditions, resulting in higher K<sub>M</sub> values (see TABLE ONE).

From both kinetic data we calculated the amount of ATP molecules required to unfold one GFP11 molecule. In the case of VATΔN, the number of ATP molecules was similar for both Mg<sup>2+</sup> concentrations (140 for low and 112 for high Mg<sup>2+</sup>), but the consumption of ATP by VAT to unfold one GFP11 molecule was much higher and strongly dependent on the Mg<sup>2+</sup> concentration (1511 for low and 426 for high Mg<sup>2+</sup>). At a concentration of 20 mM Mg<sup>2+</sup> VAT worked very inefficiently and consumed nearly four times the amount of ATP molecules than at 120 mM to unfold one GFP11 molecule. The substrate did not account for the increase of the ATP hydrolysis by VAT; since neither the ATPase activity of VAT nor VATΔN was stimulated by GFP11.

**The Unfolding of Non-tagged GFP—**Surprisingly, we detected unfolding of non-tagged, i.e. native GFP by VAT and VATΔN, but with lower efficiency as compared with GFP11 unfolding. The rate of unfolding by VATΔN was again higher than that of VAT, and VAT showed unfolding only at high Mg<sup>2+</sup> (Fig. 1C). This is a novel finding, because hitherto none of the studied AAA<sup>+</sup> ATPases has been shown to be able to unfold native GFP, i.e. without ssrA tag. The crystal structure and mutational analysis of GFP revealed that the C-terminal 9 residues are unstructured and dispensable for fluorescence (40). Our results show that the
unstructured 9 amino acid residue tail of native GFP is sufficient for recognition and unfolding by VAT and implies that conformational properties like the absence of structure, rather than a specific sequence, are critical for VAT-induced unfolding. This assumption is supported by the observation that both VAT and VAT\(\Delta N\) unfolded a GFP mutant protein, where the unstructured 9-amino acid residue tail was deleted and replaced by the ssrA-tag (data not shown).

**Substrate Interaction through D1**—Residues of the central channel are conserved in many AAA\(^+\) proteases (Fig. 2C), they line the pore of the hexameric complexes (Fig. 2D) and were shown to be involved in substrate binding (41).

The binding of substrates to VAT\(\Delta N\), lacking the N domain, must occur either via the D1 or the D2 domain. To identify the domain responsible for substrate binding and unfolding, we mutated conserved aromatic or rather hydrophobic residues in the pore sections of D1 or/and D2 of VAT and measured the unfolding activity of the isolated mutant VAT proteins (Fig. 2A). We show here that the pore residue tyrosine 264 of VAT-D1 is an essential residue for substrate binding.

### TABLE ONE

**Comparison of kinetic constants of ATP hydrolysis and GFP11 unfolding**

| Kinetic Constants | VAT (20 mM Mg\(^{2+}\)) | VAT (120 mM Mg\(^{2+}\)) | VAT\(\Delta N\) (20 mM Mg\(^{2+}\)) | VAT\(\Delta N\) (120 mM Mg\(^{2+}\)) |
|-------------------|--------------------------|--------------------------|-------------------------------|-------------------------------|
| \(V_{\text{max}}\) in nmol Pi/μg \(k_{\text{cat}}\) in s\(^{-1}\) | 0.27 ± 0.01 | 2.59 ± 0.05 | 6.49 ± 0.07 | 5.27 ± 0.07 |
| \(K_{m}\) in mM | 0.22 ± 0.01 | 0.35 ± 0.02 | 0.13 ± 0.01 | 0.10 ± 0.01 |
| \(k_{\text{cat}}/K_{m}\) | 22 | 41 | 33 | 33 |
| ATP/GFP11\(^a\) | 1511 | 140 | 112 | 112 |

\(^a\) Molecules ATP hydrolyzed per molecules of GFP11 unfolded.
VAT Is Regulated by Its N Domain

The ATP hydrolysis of both mutant proteins was strongly reduced, by 90% for B1 and by 70% for B2, and the sum of the residual ATPase activities of both mutants adds only up to 40% of the activity of wild-type VAT (compare VAT, B1, and B2 in Fig. 4B). This indicates that ATP hydrolysis by wild-type VAT is an interdomain cooperative reaction, as reported for mammalian p97 and yeast Hsp104 (35, 42). Our findings demonstrate that the VAT-D1 domain, despite having only 30% of the ATPase activity as compared with wild-type VAT, is an efficient unfolding domain (B2 mutant). When ATP hydrolysis in the D1 domain was inactivated (B1 mutant), the D2 domain unfolded the substrate with a lower rate but with the same efficiency as the D1 domain (B2 mutant) (40% decreased unfolding but similarly decreased ATPase activity). The ATP hydrolysis of none of the mutants was stimulated by addition of GFP11.

Mutations of the sensor-1 residues (N334A in D1 and N612A in D2) had a slightly more pronounced effect on ATP hydrolysis and GFP11 unfolding than mutations of the Walker B residues (compare S1 and B1 and S2 and B2 in Fig. 4). Different functions have been described for sensor-1 residues, such as sensing the nucleotide state of the protein (43, 44) or catalyzing the hydrolysis of ATP via coordination of a water molecule (42, 45, 46). Our site-directed mutagenesis results indicate that the sensor-1 residue in both domains of VAT is equally important for ATP hydrolysis as the respective catalytic Walker B residue. Therefore it is very likely that both sensor-1 residues in VAT have a catalytic function in ATP hydrolysis. In accordance with this, the double mutant VAT-N334/N612A does not have any ATP-hydrolyzing activity (SS in Fig. 4).

Remarkably, when ATP binding to the second domain was eliminated by exchange of the Walker A box lysine 514 for alanine (see supplemental Fig. S4), the unfolding activity of D1 was reduced to 35% of the wild-type activity (VAT-K514A; A2 in Fig. 4). Since the Walker B ...
mutation E568A in D2 of VAT did not reduce GFP11 unfolding by D1, we conclude that nucleotide binding in D2 promotes efficient unfolding by D1. Vice versa, exchange of the Walker A box lysine 237 for alanine in the first domain led to a complete loss of substrate unfolding activity (VAT-K237A; A1 in Fig. 4), which is consistent with the need of nucleotide binding in D1 for an efficient substrate interaction (Fig. 3).

**DISCUSSION**

In the studies described above, we demonstrate for the first time directly the unfolding activity of a globular protein by a member of the p97/VCP protein family. While for eukaryotic p97/VCP complexes evidence for the extraction of unfolded proteins from the endoplasmic reticulum was recently presented (9, 47–49), we report here the unfolding of the globular GFP protein by the archaeal homologue VAT.

We describe stimulation of GFP11 unfolding activity of VAT by Mg\(^{2+}\), resulting from enhanced ATP hydrolysis of VAT at high Mg\(^{2+}\) conditions. In contrast, the ATP hydrolysis and also GFP unfolding of VAT\(\Delta N\), an N domain deletion mutant of VAT, is higher than that of VAT and not stimulated by high Mg\(^{2+}\) conditions. Recent studies have shown that p47 binds to the N domain of p97 (50, 51), and no binding occurs to the mutant p97 deleted for the N domain (48). The cofactor binding leads to a decrease in ATP hydrolysis of wild type p97 (18). For the related eukaryotic NSF protein the modulation of ATPase activity by the N domain was also reported. Upon binding of the cofactor ω-SNAP to the N domain of NSF its ATPase activity is enhanced (13, 19). Thus, the regulation of the ATPase activity of eukaryotic NSF and NSF as well to the N domain of NSF its ATPase activity is enhanced (13, 19). Thus, the related eukaryotic NSF protein the modulation of ATPase activity by the mutant p97 deleted for the N domain (48). The cofactor binding leads to a decrease in ATP hydrolysis of wild type p97 (18). For the related eukaryotic NSF protein the modulation of ATPase activity by the N domain was also reported. Upon binding of the cofactor ω-SNAP to the N domain of NSF its ATPase activity is enhanced (13, 19).

The Mg\(^{2+}\) sensitivity of VAT suggests that high Mg\(^{2+}\) assay conditions might have induced conformational changes in the protein, which abrogated the regulatory and/or inhibitory function of the VAT-N domain on the ATPase activity and simultaneously facilitated access to the substrate-binding sites. Nucleotide-dependent movements of the N domain have been visualized by electron microscopy and crystal structure analysis of mammalian p97 (33, 52–54).

Performing site-directed mutagenesis of highly conserved hydrophobic pore residues in D1 or/and D2 of VAT and measuring the unfolding activity of the mutant proteins, we found the pore residue tyrosine 264 in D1 to be essential for substrate binding (Fig. 3) and unfolding (Fig. 2). Unexpectedly, the ATPase activity of this mutant was significantly increased. This fact could result from higher flexibility in the pore region due to a smaller residue, as reported for HsU (55). Alternatively, the mutation of the aromatic residue might mimic the interaction with substrate and stimulate the ATPase activity of VAT permanently, as found for other AAA ATPases. However, for our model substrates GFP and GFP11, a stimulation of the ATPase activity could not be demonstrated. But compared with p97 (18, 34) and other AAA ATPases (56, 57), VAT shows strongly stimulated ATP hydrolysis under 120 mM Mg\(^{2+}\) assay conditions. The ATP hydrolysis rate of VAT at 20 mM Mg\(^{2+}\) is in the same range as that of p97, but in contrast to VAT, the ATPase activity of p97 is inhibited by higher Mg\(^{2+}\) conditions (34). The low ATPase activity and the lack of stimulation by Mg\(^{2+}\) might explain why GFP11 unfolding was not detected with p97.2 Notably, the unfolding activity of VAT at 20 mM Mg\(^{2+}\) is very low.

Mutation of the pore residue tryptophane 541 in D2 also had an inhibitory effect on GFP11 unfolding, but compared with the effect of exchanging tyrosine 264 in D1, the inhibition was modest. Simultaneous mutation of two adjacent D2 pore residues in VAT-W541A/V542A blocked the GFP unfolding almost completely. The involvement of these residues in GFP11 binding and unfolding might suggest a vectorial translocation through both sections of the pore. Distinct from ClpP, where both pore residues are located near the entrances of the central channel (58), the pore residues of the D2 domain of p97 are in the center of the channel and are therefore most likely unable to mediate initial binding of substrates (see Fig. 2D).

Recently, the crystal structure of mouse p97 revealed a Zn\(^{2+}\) ion in the center of the pore of the D1 domain, which is coordinated via the histidine 317 residues of all six subunits. This led to the suggestion that p97 does not thread substrates through its pore (32). However, the Zn\(^{2+}\) ion was only found in the presence of ADP or the transition state mimic ADP-AIF3, but neither in the presence of AMP-PNP (33) nor in three related crystal structures of p97 N-D1 and full-length p97 (51, 59, 60). Moreover, histidine 317 is not conserved in Thermoplasma VAT or yeast Cdc48, which have a glutamine or asparagine at the respective position and thus unlikely coordinate a cation. Addition of Zn\(^{2+}\) ions (25 μM) did not inhibit GFP11 unfolding by VAT (data not shown). Therefore, the unfolding of substrate proteins by VAT is consistent with a threading mechanism through the central channel (Fig. 5). Such a threading (61) or pore feeding mechanism (54) was suggested for p97 earlier, although a concurrent model suggests unfolding without passage through the pore (32, 33).

The analysis of point mutants of the catalytic ATPase residues should help in finding the domain responsibilities in VAT. The Walker B variant VAT-E568A (D2 mutant) showed the same unfolding activity of GFP11 as wild-type VAT, demonstrating that the energy provided by D1 domain-mediated ATP hydrolysis is sufficient for GFP unfolding. On the contrary, the GFP unfolding of VAT-E291A (D1 mutant) was reduced to 40% of wild-type activity. Significantly, the ATP hydrolysis of both mutants was strongly reduced, and the total activity was less than that of wild-type VAT, which suggests interdomain cooperative ATP hydrolysis. This was described for other AAA ATPases, e.g. yeast cytoplasmic Hsp104 (42) and mitochondrial Hsp78 (62), mammalian p97 (35), and *Thermus thermophilus* ClpB (57). The mutations of the sensor-1 residues (N334A and N612A) had an effect on ATP hydrolysis and GFP11 unfolding comparable with mutations of the Walker B residues, indicating an equally important function for ATP hydrolysis.

When ATP binding to the second domain was eliminated by mutation of the Walker A box lysine 514 (VAT-K514A), the unfolding activity of D1 was reduced to 35% of the wild-type activity. Vice versa, exchange of the Walker A box lysine 237 in the first domain (VAT-K237A) led to a complete loss of substrate binding and unfolding.

Thus GFP11 unfolding is dependent on both the hydrophobic D1 pore residue and nucleotide binding in D1. Therefore we propose a model where VAT catalyzes unfolding of a globular protein via a thread-
ing mechanism (Fig. 5), which was demonstrated before for several other AAA+ ATPases but not for the eukaryotic p97 or NSF proteins. Although VAT is most likely the evolutionary precursor of these proteins, the eukaryotic descendants have evolved and might have developed distinct mechanisms for protein remodeling.

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