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Mutations in p97/VCP induce unfolding activity

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Abstract **A comparison of the protein sequences of various two-domain AAA+ ATPases revealed a striking difference in the residues lining the central pore of the D1 domain. The protein unfoldases of the bacterial Clp family and the archaeal VAT protein have at least one aromatic residue in the central D1 pore. In contrast, none of the members of the eukaryotic p97/VCP protein family has an aromatic residue in the D1 pore. The protein unfolding activity of VAT and other AAA+ ATPases is critically dependent on the presence of aromatic residues in this central pore. Unfoldase activity has not been demonstrated for the p97/VCP family in vitro. Thus, we exchanged the two aliphatic residues leucine and alanine of the D1 pore for aromatic tyrosine residues in full length p97 and in p97ΔN, a truncated form of p97 lacking the N domain. We found that the mutant p97ΔN variants with a single tyrosine or with two tyrosine residues in the central pore of D1 unfold the Clp family and VAT model substrate YFP-ssrA, whereas full length p97 with aromatic pore residues and wild-type p97 or p97ΔN do not. Thus, p97 can exert unfoldase activity in vitro, provided that a single tyrosine residue is introduced into the D1 pore and that the N domain is deleted.**
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1. Introduction

The eukaryotic p97/VCP protein is member of the ubiquitously distributed ATPase associated with various cellular activities (AAA+) family of proteins, for recent reviews see [1,2] and two special issues of the Journal of Structural Biology on AAA+ proteins [3,4]. Mammalian p97/VCP and its yeast homolog Cdc48 are involved in diverse cellular processes [5–8]. Amongst these processes one important function is protein quality control in the endoplasmic reticulum-associated degradation pathway of protein degradation [9–11]. The extraction of proteins from the ER is energy-dependent and requires the function of p97 [12], which is a hexameric, barrell-shaped protein complex built of six identical 90 kDa subunits [13–16]. The polypeptide chain is comprised of three domains, an N-terminal domain followed by two consecutive AAA domains, D1 and D2. The hexameric complex forms a central pore which traverses the whole complex and is lined by three conserved loops, one in D1 and two in D2. The residues of the two loops in the D2 domain have been shown to be important for substrate processing [17]. Residues corresponding to the loop in the D1 domain of p97 have been mutated in VAT, the archaeal ancestor of p97, and have been demonstrated to be essential for unfolding of the model substrate YFP-ssrA [18]. Corresponding residues in the loops of other AAA+ unfoldases were also shown to be essential for protein unfolding.

Strikingly, the loop in the p97 D1 domain does not contain any aromatic residue, in contrast to VAT and other AAA+ unfoldases. Since wild-type p97 and the truncated variant p97ΔN could not unfold YFP-ssrA, we introduced aromatic residues into the loop of the D1 domain. We found that the introduction of one or two aromatic residues into the D1 pore of p97ΔN, but not full length p97, induced the unfolding of YFP-ssrA.

2. Materials and methods

2.1. Site directed mutagenesis

Expression vectors for the human p97 and p97ΔN pore mutants were obtained by site directed mutagenesis of pPRO-EX-HTa/p97 (residues 1–806) and pPRO-EX-HTa/p97ΔN (residues 200–806) and pPROEXHTa/p97N199 (residues 1–199) using the Stratagene QuikChange Site-Directed Mutagenesis Kit with *E. coli DH5α* as host cells. The following sense (s) and antisense (as) primer pairs were used to introduce the respective mutations in the D1 domain of p97:

D1YY s 5′-GGTCCTGAGATCATGAGCAAATactaTGGT-GAGTCTGAGAGCAACC-3′; as 5′-GGTTGCTCTCAGACTCAC-CAtagtATTTGCTCATGATCTCAGGACC-3′; D1YA s 5′-GGT CCTGAGATCATGAGCAAATacGCTGGTGAGTCTGAGAGC-AACC-3′; as 5′-GGTTGCTCTCAGACTCACCAGCgtATTTGCT-CATGATCTCAGGACC-3′; D1LY s 5′-GGTCCTGAGATCAT-GAGCAAATTGtaTGGTGAGTCTGAGAGCAACC-3′; as 5′-GG-TTGCTCTCAGACTCACCAtaC AATTTGCTCATGATCTCAG-GACC-3′.

2.2. Protein expression and purification

The His_6-TEV-constructs of the p97 and p97ΔN pore mutants were expressed in *E. coli* BL21 (DE3). Cells were grown at 37 °C to an OD_{600} of 0.8. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After 4 h cells were harvested by centrifugation (15 min at 4500 × *g*), washed with 50 mM $NaPO_4$, pH 8.0; 300 mM NaCl and frozen at −20 °C. To purify the recombinant proteins, cells were lysed by thawing, lysozyme (Sigma) treatment (1 mg/ml for 30 min at 4 °C) and sonication (5 min at 50% duty cycle with a Branson Sonifier 250). DNA and RNA were digested (10 μg/ml RNase A (Sigma) and 5 μg/ml DNase I (Sigma) for

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Abbreviations: AAA, ATPase associated with various cellular activities; NSF, *N*-ethylmaleimide-sensitive factor; VCP, valosin-containing protein; YFP, yellow fluorescent protein

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15 min on ice) and cell debris was removed by centrifugation (30 min at $11\,500 \times 9g$). The recombinant proteins were then purified from the supernatant by Ni-NTA affinity chromatography on a 1 ml *HisTrap HP* column (Amersham Biosciences). The column was equilibrated, loaded and washed with 50 mM $NaPO_4$, pH 8.0; 300 mM NaCl; 20 mM imidazole. The proteins were eluted with a continuous gradient of 20–400 mM imidazole. Further purification was achieved by gel filtration using a Superose 6 XK 16/70 (Amersham Biosciences) run with 50 mM HEPES/NaOH, pH 7.5; 100 mM NaCl; 5 mM $MgCl_2$. The His_6-Tag was removed by incubating the purified protein for 3 h at 30 °C with recombinant His_6-tagged TEV protease in a ratio of 1 mg TEV protease per 5 mg of the purified protein. The untagged protein was obtained in the flow-through of a 1 ml HisTrap HP Ni-NTA column (Amersham Biosciences) and dialyzed against 50 mM HEPES/NaOH, pH 7.5; 100 mM NaCl; 5 mM $MgCl_2$. For storage 1 mM DTT and 10% glycerol were added.

2.3. Electron microscopy

The electron micrographs of negatively stained (2% uranylacetate) p97 after Superose XK 16/70 purification were recorded with a Philips CM 200 FEG transmission electron microscope at 160 kV.

2.4. ATPase assay

ATPase activity was determined by incubating the p97 proteins at a concentration of 100 nM in 50 mM Tris–Cl, pH 7.5; 10 mM or 120 mM $MgCl_2$; 1 mM DTT and 10 mM ATP in a volume of 50 μl at various temperatures as indicated in the figure legends. The reaction was allowed to proceed for 4 min before adding 800 μl malachite green solution [19], incubating for 1 min at room temperature and adding 100 μl of 34% citric acid. After another 30 min, the OD_{640} was measured and used to calculate the concentration of inorganic phosphate from a calibration curve.

2.5. Protein unfolding assay

The unfolding of YFP-ssrA was monitored by the loss of fluorescence using a Fluostar Optima spectrofluorometer (*bmg*). Concentration of proteins was: 100 nM p97 protein (hexamer) or its mutants, 25 nM *Thermoplasma* 20 S αΔN proteasome (deleted for the N-terminal peptides of the α subunits [20]) and 500 nM YFP-ssrA. Proteins were incubated in a volume of 100 μl of 50 mM Tris–Cl, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT in a 96-well-plate at 37 °C for 10 min. The unfolding reaction was started by adding 10 mM ATP and the change of fluorescence was recorded using filters of 480 nm for excitation and 520 nm for emission.

3. Results

3.1. The unique pore motif in the p97 D1 domain

During our studies of the *Thermoplasma* VAT protein, the archaeal homolog of p97, we noticed that the residues forming the central pore in the D1 domain of VAT are distinct from the residues in p97 (Fig. 1). VAT and most archaeal homologs share the motif K**YY**G, whereas in the p97 family the two aromatic tyrosine residues are exchanged for non-aromatic residues, e.g. K**LA**G in mammalian p97, K**MA**G in yeast Cdc48 or K**MS**G in the two homologs found in *C. elegans*. In contrast, the residues forming the central pore in the D2 domain of p97 and VAT are similar, with two or one aromatic residues, respectively. The consensus for the D2 pore is M**WF**G in the p97 family and K**WV**G in the VAT family. The respective pore residues of several AAA+ ATPases involved in protein unfolding, i.e. *E. coli* ClpA, ClpB, ClpX, HslU, FtsH and yeast Hsp104, contain at least one aromatic residue (see Fig. 2 in [18]). Site directed mutagenesis of the respective aromatic residues in VAT and the AAA+ ATPases mentioned above has demonstrated that these residues are essential for the unfolding of substrate proteins [18]. Interestingly, the model substrate YFP-ssrA was unfolded by VAT but not by p97.

Ta-VAT-D1	GPEIMSKYYGQSEQKL
Hs-p97-D1	GPEIMSKLAGESESNL
Sc-Cdc48-D1	GPEVMSKMAGESESNL
Ce-C41C4.8-D1	GPEIMSKMSGESESNL
Hs-NSF-D1	GPEILNKYVGESEANI
Ta-VAT-D2	GPEVLSKWVGESEKAI
Hs-p97-D2	GPELLTMWFGESEANV
Sc-Cdc48-D2	GPELLSMWYGESESNI
Ce-C41C4.8-D2	GPELLTMWFGESEANV
Hs-NSF-D2	SPDKMIGFSETAKCQA

Fig. 1. Comparison of the pore region from p97 and homologs. Protein sequence alignment of the region around the pore residues of the D1 and the D2 domain, highlighted in green for aromatic and in red for aliphatic pore residues. Notably the D1 pore of p97 does not contain aromatic residues, in contrast to its own D2 pore as well as both pores of *Thermoplasma* VAT and human NSF. Abbreviations are: Ce, *Caenorhabditis elegans*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; and Ta, *Thermoplasma acidophilum*.

Therefore, we decided to test the function of the two central pore residues in the human p97 D1 domain by exchanging them for aromatic tyrosine residues yielding the pore motifs K**YY**G (as present in VAT), K**YA**G and K**LY**G.

3.2. The p97 and p97ΔN D1 pore mutants form hexameric complexes

We had found in our studies of VAT that the deletion of the N domain increased the ATPase and the GFP-ssrA unfolding activity of the protein [18]. Therefore, we introduced tyrosine residues into D1 of full length p97 and the corresponding p97ΔN mutant by site-directed mutagenesis. This yielded the p97D1YY and p97ΔND1YY proteins, both sharing the same D1 pore motif K**YY**G as found in VAT. The p97D1YY and p97ΔND1YY proteins and the corresponding p97 and p97ΔN variants with the wild-type pore motif K**LA**G were produced with an N-terminal His_6-TEV-tag in *E. coli* and purified by Ni-NTA affinity and size exclusion chromatography (Fig. 2A). The N-terminal His_6-TEV-tag was removed by digesting the purified protein with the TEV protease. Like p97 and p97ΔN, the pore mutant proteins p97D1YY and p97ΔND1YY form hexameric complexes. Assembly was confirmed by gel filtration chromatography (data not shown), native-polyacrylamide gel electrophoresis (Fig. 2B) and electron microscopy (Fig. 2C and D). Similar results were obtained for the single pore mutants (data not shown). These results demonstrate that pore residues do not interfere with the ability of p97 to form hexameric complexes.

3.3. Characterization of the ATPase activity of p97 proteins

Having confirmed that the mutant proteins assemble into hexamers, we analyzed their ATPase activities at different temperatures between 37 °C and 61 °C using the malachite green method (Fig. 3A). The maximal ATPase activity for both the full length pore mutant and its N-terminal deletion variant was found at 45 °C. Throughout the tested temperature range, the ATPase activity of p97ΔND1YY is significantly increased compared to the full length protein. Obviously, the N-terminal domain exerts an inhibitory effect on the ATPase activity. The

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