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# IBMPFD and p97, the Structural and Molecular Basis for Functional Disruption

Wai-Kwan Tang and Di Xia

*Laboratory of Cell Biology,  
Center for Cancer Research, National Cancer Institute,  
National Institutes of Health, Bethesda, Maryland,  
USA*

## 1. Introduction

Inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD, OMIM 167320) is an inherited, autosomal dominant, adult onset multi-disorder, which affects the muscle, bone and the brain (Watts et al., 2004). It is a rare condition with unknown worldwide prevalence. Affected individuals may display one or a combination of the following three symptoms which, however, are generally not recognized until patients are in their 40s or 50s (Weihl, 2011). (1) IBM (Inclusion body myopathy): About 90% of all patients develop proximal and distal muscle weakness initially with atrophy of the pelvic and shoulder girdle muscles (Kimonis et al., 2000; Kimonis et al., 2008b; Kovach et al., 2001; Watts et al., 2003). Cellular inclusion bodies and rimmed vacuoles are commonly found in these muscle tissues (Kimonis et al., 2008a; Kimonis et al., 2000; Watts et al., 2004). Characteristically, two proteins are most frequently found co-localized with the inclusion, ubiquitin and TDP-43 (TAR DNA binding protein-43) (Ritson et al., 2010; Weihl et al., 2008). Ubiquitin is a signaling molecule that directs protein substrates into a variety of cellular pathways, including protein degradation. Misfolded or unwanted proteins are labeled with ubiquitin, mostly in the form of polyubiquitin, and targeted for degradation (Clague and Urbe, 2010). TDP-43, on the other hand, is believed to be a substrate itself for either proteasome or autophagal degradation (Caccamo et al., 2009; Wang et al., 2010). Detection of these proteins in the inclusions suggests impairments in the protein degradation pathways. (2) PDB (Paget's disease of the bone): About half of IBMPFD patients develop PDB, which is caused by an imbalance in the activities between osteoblasts and osteoclasts. (3) FTD (Frontotemporal dementia): Only ~30% of patients develop FTD, which is characterized by language and/or behavioral dysfunction. Interestingly, clinical manifestation of these symptoms is rather random, and has no clear-cut correlation with family history or mutations. Even within isolated families bearing the same genetic mutation, individuals can exhibit different symptoms. These heterogeneities in clinical presentations cause frequent misdiagnoses of IBMPFD patients (van der Zee et al., 2009). Accurate diagnosis of IBMPFD often requires molecular genetic testing, in addition to a combined clinical diagnosis of myopathy, PDB and FTD.

## 2. What is p97?

In the year 2000, IBMPFD was recognized as a genetically distinct clinical syndrome (Kimonis et al., 2000) and was subsequently linked to heterozygous missense mutations in a highly abundant cellular protein called p97 (also called valosin-containing protein, VCP) (Watts et al., 2004). P97 belongs to the family of AAA<sup>+</sup> proteins (ATPases Associated with various cellular Activities), which use the energy from hydrolyzing ATP to drive mechanical work necessary for a host of functions including homotypic membrane fusion, cell cycle regulation and protein degradation (Wang et al., 2004; Woodman, 2003; Ye, 2006). The multi-functionality of p97 is consistent with its embryonic lethality when the p97 gene or its homologs are disrupted or knocked-out in the mouse, in yeast, in trypanosomes, and in *Drosophila* (Frohlich et al., 1991; Lamb et al., 2001; Leon and McKearin, 1999; Muller et al., 2007). Moreover, the functional versatility of p97 appears to lie in its ability to interact with a large variety of adaptor proteins. For instance, binding to the protein p47 incorporates p97 in the membrane fusion pathway (Kondo et al., 1997), whereas the p97-Ufd1-Npl4 complex participates in ER associated degradation (ERAD) (Richly et al., 2005). So far, more than twenty adaptor proteins have been identified that interact with p97 (Madsen et al., 2009), but detailed molecular mechanisms of these interactions remain elusive.

## 3. Structure of p97

Structurally, p97 is a homo-hexamer, each subunit (806 residues) consisting of three domains: a unique N-terminal domain (N-domain) followed by two conserved AAA<sup>+</sup> ATPase domains (D1- and D2-domain) in tandem (DeLaBarre and Brunger, 2003; Huyton et al., 2003) (Fig. 1A). The N-domain (residues 1-184) contains two sub-domains, an N-terminal double  $\Psi$ -barrel and a C-terminal four-stranded  $\beta$ -barrel, and is responsible for interacting with most adaptor proteins as well as with protein substrates. Both the D1- (residues 211-463) and D2-domains (residues 483-762) are typical AAA<sup>+</sup> ATPase domains comprised of two sub-domains: a large N-terminal RecA-like domain with an  $\alpha/\beta$  fold and a smaller C-terminal  $\alpha$ -helical bundle domain. The D1-domain is essential for hexamerization of p97 subunits (Wang et al., 2003) and the hexameric ring formation is predominantly mediated through interactions between the RecA-like sub-domains (Fig. 1B). However, unlike many members of the AAA<sup>+</sup> family proteins such as the *E. coli* ClpA unfoldase, the hexamerization of p97 subunits does not require the binding of nucleotide (ADP or ATP) at D1-domains, though it has been shown that nucleotide binding does accelerate p97 hexamer formation (Singh and Maurizi, 1994; Wang et al., 2003). Most of the ATPase activities of p97 involve the D2-domain, presumably required for the processing of substrates (Song & Li, 2003).

Connecting the domains are loops that have been shown to play important functions. The N-D1 loop is 27 residues long and is embedded at the interface between the N-domain and the D1-domain. The short peptide stretch (residues 763-806) immediately following the D2-domain is another region for adaptor protein binding. Although not as common as the N-domain, this C-terminal tail has been shown to interact with a number of proteins, such as Ubxd1 (Allen et al., 2006; Madsen et al., 2008). Similar to other Type-II AAA<sup>+</sup> assemblies, the p97 assembly was revealed by electron microscopy (EM) and crystallography to be a two-tiered concentric ring encircling a central pore or axial channel (Fig. 1B). The N-domains are

attached to the periphery of the D1 ring, making one ring appear larger than the other. The central pore does not run unrestricted through the hexamer, but has a narrowing or a constriction point that is formed by a bound zinc ion (DeLaBarre and Brunger, 2003).

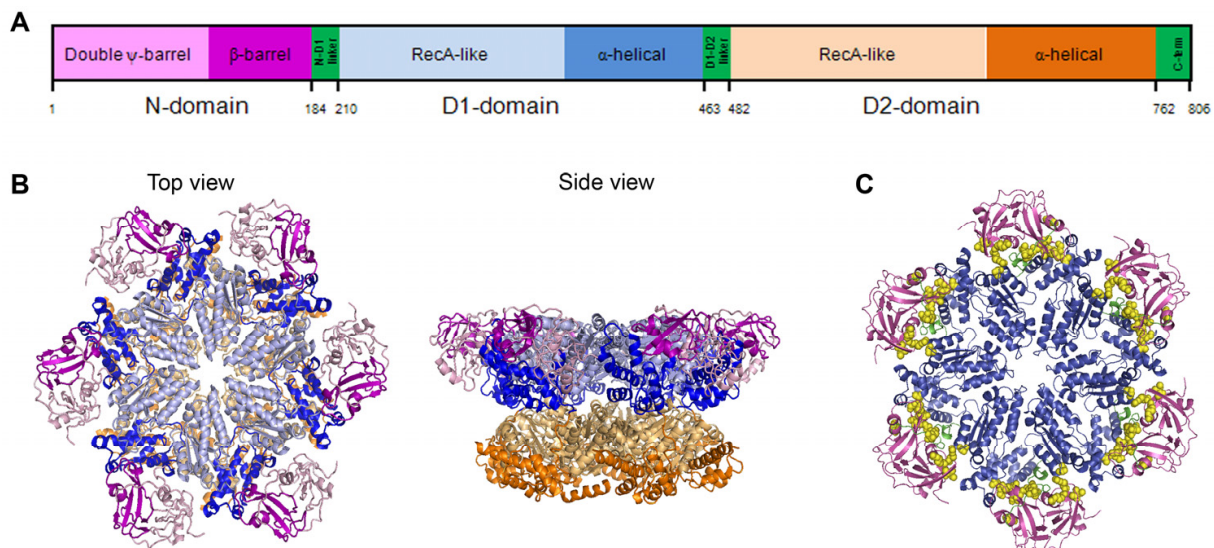


Fig. 1. **Structure of p97** (A) Domain organization of the full-length p97. (B) Ribbon representation of the crystal structure of p97 based on PDB: 1OZ4. Domains are color-coded using the scheme in (A) and two views are presented. (C) Locations of IBMPFD mutations are shown in the context of the p97 N-D1 structure (PDB: 1E32), which has the D1-domain bound with ADP. The IBMPFD mutations are represented by yellow balls. Thirteen positions representing twenty mutations are presented.

#### 4. Mutations in p97 associated with IBMPFD

So far, only single amino acid substitutions in p97 have been identified from all the clinical IBMPFD specimens examined. Altogether, twenty missense mutations found in 13 different amino acid positions in p97 have been reported to be associated with the disease and the majority of them involve substitutions of arginine residues (Table 1) (<http://www.molgen.ua.ac.be/FTDMutations>). While more than half of these mutations are located in the N-domain (Ile<sup>27</sup>, Arg<sup>93</sup>, Arg<sup>95</sup>, Pro<sup>137</sup>, Arg<sup>155</sup>, Gly<sup>157</sup> and Arg<sup>159</sup>), a few are found in the N-D1 linker region between the N- and D1-domains (Arg<sup>191</sup> and Leu<sup>198</sup>) and in the D1-domain (Ala<sup>232</sup>, Thr<sup>262</sup>, Asn<sup>387</sup> and Ala<sup>439</sup>). None has been found in the D2-domain. Among these, mutations at Arg<sup>155</sup> are the most frequently observed in patients (Table 1). Interestingly, mapping these mutations onto the three-dimensional p97 structure in the ADP-bound form revealed that they all clustered at the interface between the N- and D1-domains (N-D1-interface, Fig. 1C).

#### 5. Wild type vs. IBMPFD mutant p97: Structural characteristics

Changes in structure as a result of amino acid mutations can lead to a global disruption of the protein folding, resulting rapid clearance by cellular stress response mechanisms, or to localized structural changes that cause complete loss of the protein function, or to subtle conformational changes that alter the function of the protein. Structural changes in mutant

proteins can be directly visualized by X-ray crystallography, NMR (nuclear magnetic resonance), and EM (electron microscopy) or inferred indirectly by biophysical and biochemical methods such as SAXS (small angle X-ray scattering). To understand how IBMPFD mutations lead to functional change in p97, it is absolutely necessary to know what structural changes these mutations entail. However, knowing what has changed in mutants depends heavily on our baseline knowledge of the wild type proteins.

	Change in amino acid	Change in gene	Location	References
1	I27V	79 A → G	N-domain	(Rohrer et al., 2011)
2	R93C	277 C → T		(Guyant-Marechal et al., 2006; Watts et al., 2004)
3	R95C	283 C → T		(Kimonis et al., 2008a)
	R95G	283 C → G		(Watts et al., 2004)
4	P137L	410 C → T		(Palmio et al., 2011; Stojkovic et al., 2009)
5	R155C	463 C → T		(Gidaro et al., 2008; Guyant-Marechal et al., 2006; Schroder et al., 2005; Watts et al., 2004)
	R155H	463 C → A		(Viassolo et al., 2008; Watts et al., 2004)
	R155P	463 C → C		(Watts et al., 2004)
	R155S	463 C → A		(Stojkovic et al., 2009)
	R155L	464 G → T		(Kumar et al., 2010)
6	G157R	469 G → C		(Djamshidian et al., 2009)
		469 G → A		(Stojkovic et al., 2009)
7	R159C	475 C → T		(Bersano et al., 2009)
	R159H	476 G → A		(Haubenberger et al., 2005; van der Zee et al., 2009)
8	R191Q	572 G → C	N-D1	(Watts et al., 2004)
9	L198W	593 T → G	linker	(Kumar et al., 2010; Watts et al., 2007)
10	A232E	695 C → A	D1-domain	(Watts et al., 2004)
11	T262A	784 A → G		(Spina et al., 2008)
12	N387H	1159 A → C		(Watts et al., 2007)
13	A439S	1315 G → T		(Stojkovic et al., 2009)

Table 1. IBMPFD mutations in p97.

5.1 Conformational changes in AAA<sup>+</sup> proteins

Studies of AAA<sup>+</sup> proteins have revealed conformational changes in various domains in response to changes in the environment, to substrate binding, and to various bound nucleotides. At least some of the observed conformational changes are believed to be necessary for function (Vale and Milligan, 2000). N-domains of ClpB were found in different positions in crystal structure even though all the subunits were bound with the ATP analog AMP-PNP (Lee et al., 2003); this conformational plasticity in N-domains is likely the result of different environments each subunit experienced in the crystal and may not be directly related to function. By far most of the observed conformational changes, though relatively subtle, are induced by binding of various nucleotides in the AAA<sup>+</sup> domains. Such nucleotide-driven conformational changes have been observed for both Type I (proteins with one AAA<sup>+</sup> domain) and Type II (proteins with two AAA<sup>+</sup> domains) AAA<sup>+</sup> proteins in



crystal structures such as LTag (SV40 large tumor antigen) (Gai et al., 2004), FstH (Bieniossek et al., 2009) and HslU (Wang et al., 2001).

Conformational changes in AAA<sup>+</sup> proteins have been probed by methods other than crystallography, which do not depend on obtaining 3-D crystals albeit at relatively lower resolutions. Such methods include cryo EM and SAXS. Cryo EM has revealed flexibility in the N-domains of *E. coli* AAA<sup>+</sup> protein ClpA (Ishikawa et al., 2004) and SAXS experiments have shown large conformational changes in NtrC1 (Chen et al., 2010). Although in some cases conformational changes observed with different methods do not completely agree, it is widely accepted that AAA<sup>+</sup> proteins undergo dynamic movements during their catalytic cycle. One general observation relating to structural movements among AAA<sup>+</sup> proteins is the change in size of the axial pore where substrates enter. The “open-and-close” of the axial pore in AAA<sup>+</sup> proteins is thought to provide the mechanical force needed to pull the substrates through the pore (Kravats et al., 2011; Zolkiewski, 2006).

## 5.2 Structural studies of wild type p97

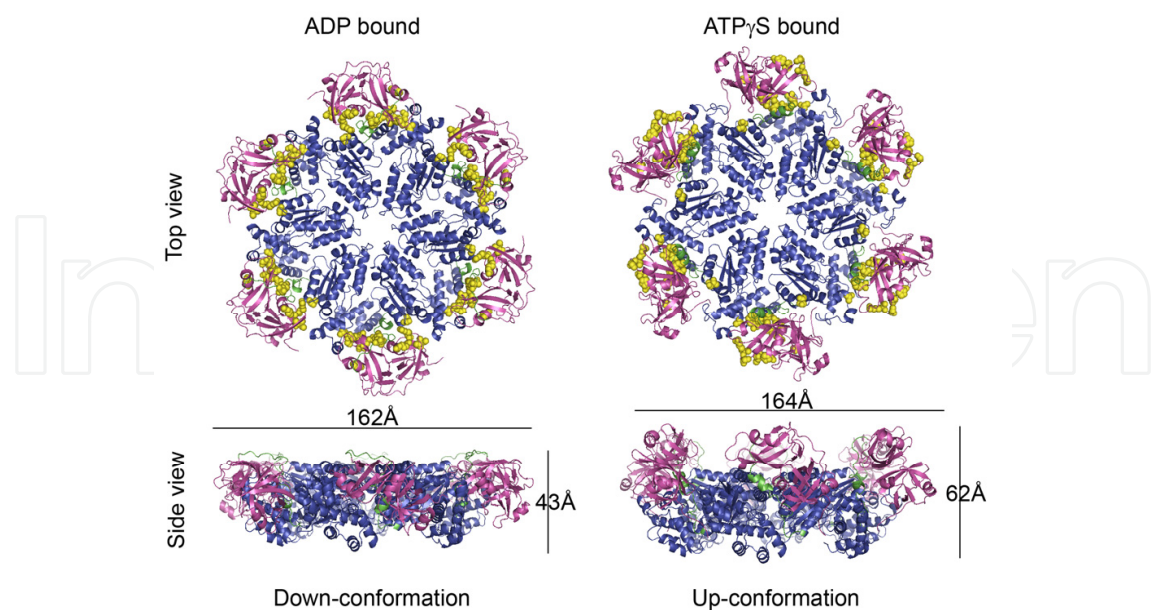
Crystal structures of wild type p97 have been solved for both the full-length protein and a truncated N-D1 fragment (absent of the D2-domain) (DeLaBarre and Brunger, 2003; Huyton et al., 2003; Zhang et al., 2000). Although both D1- and D2-domains are capable of hydrolyzing ATP (Song et al., 2003), in all the wild type p97 crystal structures determined to date, ADP was invariably found in the D1-domains, while either ADP or ADP-AlFx (transitional analog) was observed in the D2-domain (DeLaBarre and Brunger, 2003; Huyton et al., 2003). These structures share an identical N-domain conformation with the N-domains attached to the periphery of the D1 ring, and in plane with it (Fig. 1B). Unsuccessful attempts have been made to crystallize wild type p97 with other forms of nucleotides trapped in the D1-domain (DeLaBarre and Brunger, 2003).

On a different front, studies using EM and SAXS to gain structural insights into the conformational changes of p97 have revealed rather dramatic changes in the positions of N-domains (Davies et al., 2005; Rouiller et al., 2002). In contrast to X-ray crystallography, these approaches are limited to providing molecular shapes without a clear delineation of bound nucleotides. Nevertheless, large conformational changes in the N-domains of p97 can be detected by modeling individual domains from crystal structures into these molecular envelopes to re-construct structures of p97 under various conditions, although lacking absolute certainty. In the presence of different nucleotides (ATP or ADP) and their non-hydrolysable or transitional analogs (ADP-AlFx, AMP-PNP or ATP $\gamma$ S), the N-domains of p97 were shown to undergo some of the most dramatic movements during the ATP cycle. Although some changes observed by different methods or in different laboratories were not always compatible with each other, it is generally agreed that N-domains of p97 are conformationally flexible. EM studies of p97 complexed with the adaptor protein p47 also showed the N-domains undergoing a large conformational change in the presence of different nucleotides (Beuron et al., 2006). However, conflicting results were reported in these low-resolution studies on the direction of the N-domain movement in response to binding of different nucleotides. An intrinsic difficulty with these studies is the uncertainty concerning the nucleotide states at the AAA<sup>+</sup> ATPase domains due to the resolution limits of these methods. Compounding these problems, it has been known that at least half of the nucleotide-binding sites in the D1-domains of p97 are pre-occupied by ADP molecules,

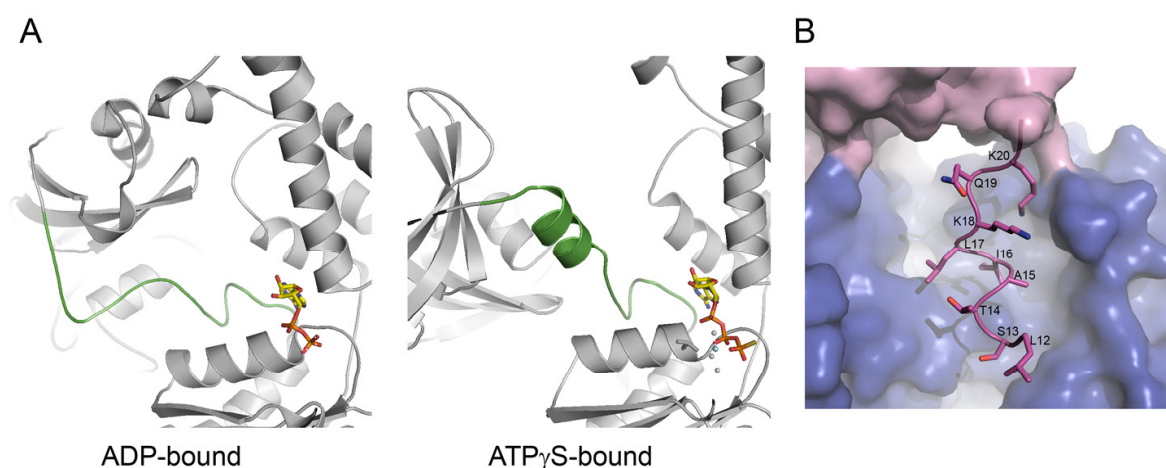
which are very difficult to remove (Briggs et al., 2008; Davies et al., 2005). Clearly, high-resolution structures of p97 in different nucleotide states are needed to unambiguously define the relationship of N-domain conformation with nucleotide-binding states.

5.3 Crystallographic studies of IBMPFD mutant p97

Recently, a new conformation of N-domains was observed by X-ray crystallography at 2 Å resolution with ATP $\gamma$ S (a non-hydrolysable ATP analog) bound at the D1-domains of two IBMPFD-associated p97 N-D1 mutants, R155H and R95G; both are N-domain mutations (Tang et al., 2010). With ATP $\gamma$ S bound at the D1-domain, the N-domain undergoes a rotational and translational movement to adopt a new position, which is in sharp contrast to the “in-plane” position with the D1-ring or Down-conformation, as observed previously in the ADP-bound form. In this new conformation, the N-domains uniformly occupy a position above the plane of the D1-ring or in the Up-conformation (Fig. 2), despite the fact that no detectable changes are seen in the N-domain itself due to either the R155H or R95G mutation (Data not shown) (Tang et al., 2010). Accompanying the transition of N-domain from the Down- to Up-conformation are two prominent structural rearrangements. One is the transition in secondary structure of the linker between N- and D1-domain (N-D1 linker), going from the random coil in the Down-conformation to the three-turn  $\alpha$ -helix in the Up-conformation reminiscent of a contracted spring pulling the N-domains out of the planar conformation upon the binding of ATP $\gamma$ S (Fig. 3A). This novel conformation of p97 demonstrated for the first time the dynamic movement of the N-domain at near atomic resolution, although observed in p97 mutants. A second change is the re-ordering of the N-terminal fragment that encompasses residues 12 to 20, which was disordered in the ADP-bound structures (Fig. 3B). This re-ordering of the N-terminal peptide apparently protects the Lys18 from limited proteolysis by trypsin seen in the ADP form of p97 (Fernandez-Saiz and Buchberger, 2010).



**Fig. 2. Changes in N-domain conformation in response to binding of different nucleotides to the D1 domain** Ribbon diagrams showing the two N-domain conformations of p97 N-D1 obtained from crystal structures. The N-domains, D1-domains and the IBMPFD mutations are colored in magenta and blue ribbons and in yellow balls, respectively.



**Fig. 3. Observed structural re-arrangements in the N-D1 linker and N-terminal peptide in response to binding of different nucleotides in the D1-domain** (A) The secondary structure of the N-D1 linker undergoes a transition from a random coil to a three-turn helix as the N-domains move from the Down- to Up-conformation. A close-up view shows the two conformations of the N-D1 linker (in green) in the ADP-bound form (Down-conformation) and in the ATP $\gamma$ S-bound form (Up-conformation) of p97 N-D1. The nucleotides are represented by sticks with carbon atoms in yellow, oxygen in red, nitrogen in blue, phosphorous in orange, and sulfur in gold. (B) The reordering of N-terminal peptide Leu<sup>12</sup> to Lys<sup>20</sup> in the ATP $\gamma$ S-bound form (Up-conformation) is represented by stick model. The rest of the N-domain and D1-domain are shown as magenta and blue surfaces, respectively.

The above observation appears to favor the hypothesis that the Up and Down movement of N-domains is nucleotide dependent because the binding of ADP at the D1-domain of p97 results in a Down-conformation while binding of ATP $\gamma$ S leads to an Up-conformation. However, this nucleotide-driven movement may be arguable, as these two conformations were observed in two different systems – the wild type in ADP form and the IBMPFD mutants in ATP $\gamma$ S form. A subsequent structure determination using the same IBMPFD mutant and ADP showed that N-domains adopt the Down-conformation, just as the wild-type p97 in the presence of ADP (Tang et al., 2010), thus unequivocally confirming the dependency of N-domain conformation on the nucleotide binding states at the D1-domain.

#### 5.4 Small angle X-ray scattering (SAXS) studies of wild type and IBMPFD mutant p97 in solution

Why crystallographic studies on wild type p97 can only reveal the Down-conformation, whereas IBMPFD p97 mutants can be crystallized in both Up- and Down-conformations was a paradox. One possible interpretation was that wild type and mutant p97 differ in nucleotide binding properties. Alternatively, this could be a crystallization effect. To investigate this, we performed SAXS experiments to identify conformational changes in IBMPFD mutants in solution. The results clearly demonstrated that in solution IBMPFD mutants undergo a nucleotide-dependent N-domain conformational change that is consistent with the Up- and Down-conformations observed in the crystals (Fig. 4). By serendipity, another major finding from this experiment was that wild type p97 also



undergoes a similar nucleotide-driven conformational change as observed in IBMPFD mutants (Fig. 4) (Tang et al., 2010). Therefore, the lack of success in crystallizing the Up-conformation of wild type p97 suggests the presence of an intrinsic conformational heterogeneity or asymmetry in the N-domains of the homo-hexamers.

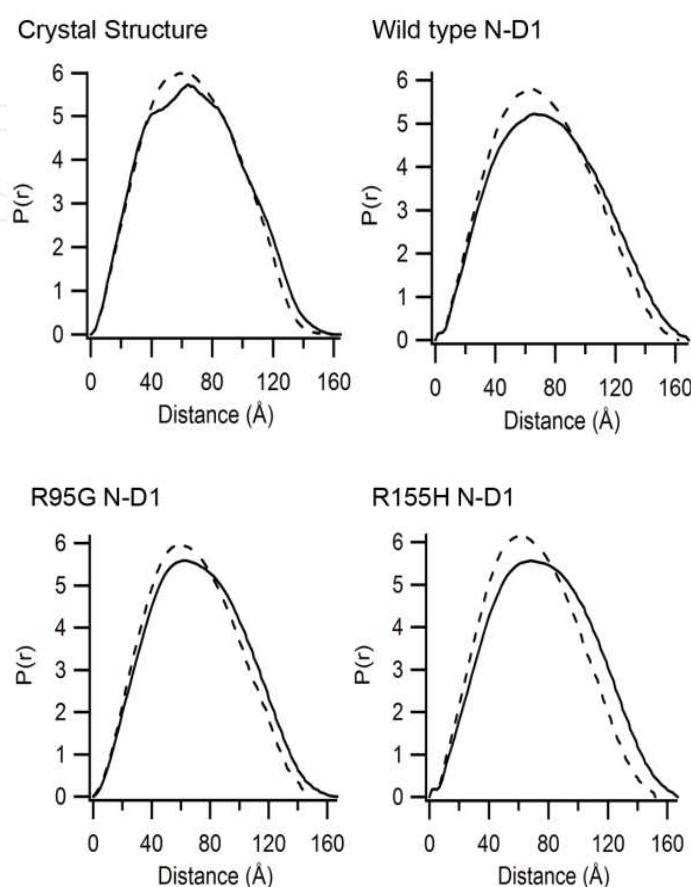


Fig. 4. **Nucleotide-driven conformational changes in solution observed by SAXS** Distance distribution functions,  $p(r)$ , of p97 N-D1 normalized to a common total probability for wild type and mutant N-D1 fragments in the presence of ADP (solid line) ADP and ATP $\gamma$ S (dashed line). The calculated distribution (Glatter, 1980) is shown of the left based on the crystal structure in the absence of bound solvent molecules.

### 5.5 ADP and ATP $\gamma$ S binding at the D1-domain

Although the binding of ATP $\gamma$ S to the D1-domain triggers a dramatic movement of the N-domain, the immediate vicinity of the D1 nucleotide-binding site shows only limited perturbations (Tang et al., 2010). When C $\alpha$  atoms of the wild type and mutant N-D1 are superimposed, the adenosine moieties of the bound nucleotides align very well and the immediate environment around the adenosine moiety shows little change. By contrast, the phosphate groups in the alignment between ADP and ATP $\gamma$ S forms differ, even though the same set of residues are involved in contacting the  $\alpha$ - and  $\beta$ -phosphate in both the wild type and mutant structures. The  $\gamma$ -phosphate in the ATP $\gamma$ S structure is stabilized by the ionic interaction with a magnesium ion (Mg $^{2+}$ , see below), by hydrogen bonds with Gln $^{348}$  and Lys $^{251}$ , by Arg $^{359}$ , an Arg finger residue from a neighboring subunit, and by two water

molecules associated with the  $Mg^{2+}$  ion (Fig. 5). Perhaps due to better diffraction resolution, in the mutant structures of p97, a  $Mg^{2+}$  ion is present in the nucleotide-binding site of every subunit. The  $Mg^{2+}$  ion is at the center of an octahedral *mer*-triaquo complex with the additional three oxo ligands coming from the side chain of the highly conserved Thr<sup>252</sup> and from the  $\beta$ - and  $\gamma$ -phosphates. The acidic residues of the DEXX sequence (Asp<sup>304</sup> and Glu<sup>305</sup>) in the Walker B motif make hydrogen bonds with two of the water molecules in the  $Mg^{2+}$  coordination sphere and, additionally, Asp<sup>304</sup> stabilizes Thr<sup>252</sup>. As expected, most of the changes in the nucleotide-binding environment are a consequence of the introduction of the  $\gamma$ -phosphate.

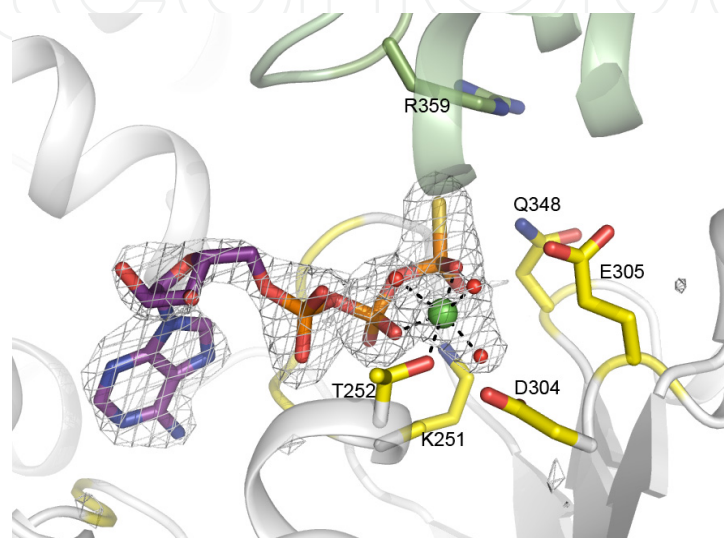


Fig. 5. **ATP $\gamma$ S binding vicinity of the D1-domain** The nucleotide-binding pocket is located at the subunit interface. The two subunits are in different colors, green and gray. The ATP $\gamma$ S molecule is shown as a stick model with carbon atoms in purple, oxygen in red, nitrogen in blue, phosphorous in magenta, and sulfur in yellow. The ATP $\gamma$ S molecule is enclosed in a difference electron density cage contoured at the 2.5  $\sigma$  level. The  $Mg^{2+}$  ion is shown as a green ball with three coordinating water molecules in red.

## 6. Wild type vs. IBMPFD mutant p97: Biophysical and biochemical characteristics

Structurally observed differences in the N-domain conformation of p97 strongly suggest a change in nucleotide binding affinities between wild type and IBMPFD mutants, even though the binding environment for nucleotide seems unperturbed in mutant p97. The fact that mutant p97 can be crystallized in the presence of ATP $\gamma$ S suggests a few competing hypotheses: one of which is the possibility of IBMPFD mutants acquiring a higher affinity for ATP $\gamma$ S, leading to an alteration in ATPase activity. Alternatively, IBMPFD mutations could lead to a reduced ADP binding affinity. From structural studies, we can readily infer that the D1 nucleotide-binding site is seriously affected by IBMPFD mutations. However, how mutations at the N-D1 interface influence the D1 or D2 ATPase sites is not clear. Measuring the binding affinities of various nucleotides toward either D1 or D2 ATPase sites and the ATPase activities of the protein will provide biochemical indications as to how IBMPFD mutations might affect the biochemical properties of p97.

6.1 Nucleotide-binding affinity and ATPase activity

As mentioned earlier, p97 has two ATPase domains, D1 and D2; both are capable of hydrolyzing ATP. ATPase activity requires the presence of  $Mg^{2+}$  and is stimulated by high temperature (Song et al., 2003). While the D2-domain mediates most ATPase activity, the D1-domain contributes to heat-induced activity (Song et al., 2003). By using isothermal calorimetry (ITC) and Walker A mutants of either the D1- or D2-domains, it was shown that the binding of both domains to ATP $\gamma$ S is similar with dissociation constants in the range of 1  $\mu$ M, but binding of ADP to the D1-domain is nearly 30-fold higher than that of the D2-domain, which is consistent with the higher ATPase activity of the D2-domain (Briggs et al., 2008).

Because the onset of IBMPFD is relatively late in life, the mutations in p97 are not expected to have dramatic defects in function. Indeed, IBMPFD equivalent mutations introduced into cdc48, a yeast homolog of p97, did not appear to interfere with normal cell growth (Esaki and Ogura, 2010). Although IBMPFD mutation sites are not in the immediate vicinity of the ATP-binding sites, as shown by crystal structures of p97, especially in the ATP $\gamma$ S bound form, reports on how mutations affect ATPase activity of p97 vary. Two reports showed that mutants exhibit higher ATPase activity than the wild type to various degrees (Manno et al., 2010; Weihl et al., 2006). One paper, by contrast, reported no significant alterations in ATPase activity of four IBMPFD mutants (Fernandez-Saiz and Buchberger, 2010). Mutant p97 also displayed an even higher level of heat-stimulated ATPase activity (Halawani et al., 2009).

ITC measurements in N-D1 fragments of p97 showed that instead of predicted higher affinity for ATP $\gamma$ S in the D1-domain, all mutants showed lowered affinity, up to five-fold weaker, towards ADP when compared to the wild type (Table 2) (Tang et al., 2010). More interestingly, all mutants displayed biphasic titration profiles toward ATP $\gamma$ S, suggesting two distinct binding sites, one high and one low affinity site.

N-D1 p97	ATP $\gamma$ S		ADP	
	$K_d$ ( $\mu$ M)	N	$K_d$ ( $\mu$ M)	N
Wild type	$0.89 \pm 0.28$	$0.12 \pm 0.01$	$0.88 \pm 0.18$	$0.35 \pm 0.06$
R95G	$0.13 \pm 0.02$	$0.56 \pm 0.01$	$2.27 \pm 0.11$	$0.62 \pm 0.08$
R155H	$0.13 \pm 0.01$	$0.61 \pm 0.01$	$4.25 \pm 0.54$	$0.72 \pm 0.18$

ITC with ATP $\gamma$ S for IBMPFD mutants showed biphasic titration curves and data were fitted with a 2-site model. The  $K_d$  values for mutants are derived from fitting to the first phase.

Table 2. Dissociation constants ( $K_d$ ) and binding stoichiometry (N) of wild type and mutant p97 N-D1 fragments for ATP $\gamma$ S and ADP determined by ITC.

6.2 Pre-bound ADP in D1 nucleotide-binding sites

A unique characteristic of p97 was demonstrated by a urea denaturation experiment; the D1 sites are occupied by a significant portion of pre-bound ADP, which is difficult to release without denaturing the protein (Davies et al., 2005). ITC experiments with wild type p97 confirmed that a fraction of D1 sites are not accessible to nucleotide titration (Briggs et al., 2008). Consistent with the lowered  $K_d$  values for ADP binding in IBMPFD mutants, D1 sites were shown to be significantly more accessible to ADP titration by ITC and displayed a

biphasic titration curve for ATP $\gamma$ S, reflecting the first phase binding of the empty sites and the second phase of the pre-bound sites (Table 2) (Tang et al., 2010). Extraction by heat denaturation experiments of pre-bound ADP from the D1 sites of p97 supported the observation by ITC that the number of titratable sites is inversely related to the amount of pre-bound ADP present at the D1-domain (Tang et al., unpublished data). These findings suggest that while in wild type p97 a significant number of sites with pre-bound ADP in D1-domains of p97 are not exchangeable by a different form of nucleotides present in solution, IBMPFD mutations have altered the environment and lowered binding affinity for ADP to allow exchange, even though the change in the ADP binding site is too subtle to be detected by crystal structures.

A consequence of lowered binding affinity for ADP in the D1-domain of IBMPFD mutants is the uniformly increased accessibility of D1 sites to various nucleotides present in solution such as ATP $\gamma$ S. Indeed, successful crystallization of mutant p97 in the presence of ATP $\gamma$ S is a result of this effect. On the contrary, the pre-bound ADP molecules in the D1-domains of some subunits of wild type p97 do not substitute for ATP $\gamma$ S, in spite of having a higher affinity towards ATP $\gamma$ S than ADP (Tang et al., 2010). Consequently, in the presence of excess ATP $\gamma$ S in solution, there will be an admixture of ADP- and ATP $\gamma$ S-bound D1 sites within a hexamer. Thus, the failure to crystallize wild type p97 in the presence of ATP $\gamma$ S is a manifestation of non-uniformity in binding to nucleotides by different subunits in the D1-domains of hexameric p97.

### 6.3 Changes in interaction with adaptor proteins

By interacting with various adaptor proteins, p97 is able to play a role in a number of important cellular pathways. Therefore, alterations in adaptor protein binding by IBMPFD mutants have been investigated in both *in vivo* and *in vitro* experiments. Again, results from different groups are not completely consistent (Fernandez-Saiz and Buchberger, 2010; Manno et al., 2010). Isolated mutant p97 exhibited the same binding as wild type p97 towards adaptor proteins p47, Ufd1-Npl4, E4B and the human UFD-2 homolog. However, mutants showed impaired binding to ubiquitin ligase E4B in the presence of Ufd1-Npl4. *In vivo* pull-down experiments using HEK293 cells showed reduced binding towards the E4B and enhanced binding towards ataxin 3, thus resembling the accumulation of mutant ataxin 3 on p97 in spinocerebellar ataxia type 3 (Fernandez-Saiz and Buchberger, 2010). However, similar *in vivo* studies were done showing enhanced binding of the Ufd1-Npl4 pair by IBMPFD mutants but not for p47 (Manno et al., 2010).

## 7. Implications concerning p97 function and disease

One major contribution of p97 to cellular processes is its apparent participation in protein quality control and homeostasis, involving the ubiquitin-proteasome degradation pathway, ER associated degradation, and formation of autophagosomes. The multifaceted clinical presentation of patients with IBMPFD is consistent with the broad spectrum of p97 functions. Indeed, pathology and the cellular hallmarks such as the accumulation of inclusion bodies and rimmed vacuoles of IBMPFD can be reproduced both in cell culture and in animal models (Custer et al., 2010; Ju and Weihl, 2010; Weihl et al., 2006; Weihl et al., 2007). However, the structural and molecular basis of how p97 is involved in these different pathways and the mechanism of how p97 mutations lead to dysfunction remain elusive.



### 7.1 IBMPFD mutations produce subtle structural and functional alteration in p97

Using structural, biophysical and biochemical approaches and through detailed comparative study of wild type and IBMPFD mutant p97, details of the molecular mechanisms of p97 at the most fundamental level are beginning to emerge. As a late onset disease, the IBMPFD mutations in p97 are not expected to dramatically disrupt cellular functions. Indeed, as shown from the cell biological, structural and biochemical data, all IBMPFD mutants (1) appear to have a normal phenotype at least in the early stage of life in cultured cells, in yeast, and in fruit flies, (2) do not have observable structural alterations in their constituent domains, as compared to the wild type, (3) can form proper hexameric ring structures, (4) have nucleotide-binding pockets indistinguishable from those of the wild type, and (5) are able to undergo nucleotide-driven conformational change in solution.

In spite of these similarities, subtle yet significant differences have also been detected in IBMPFD mutants, including (1) overall up-regulated ATPase activities, (2) ability to undergo uniform nucleotide-dependent N-domain conformational change that leads to its crystallization in the presence of ATP $\gamma$ S, (3) lowered binding affinity toward ADP in the D1-domain, (4) less non-exchangeable pre-bound ADP in the D1-domain, and (5) subtle differences in binding of adaptor proteins.

### 7.2 Asymmetry in p97 function

Enigmatic observations concerning the failure of wild type p97 to crystallize in the presence of ATP $\gamma$ S, yet being able to undergo nucleotide-dependent N-domain conformational change in solution suggest the functional importance of the non-uniform binding of nucleotides by p97 to the D1-domains and of the asymmetry in N-domain conformations among its six subunits. This asymmetry is a built-in property of wild type p97, as demonstrated by ITC and heat or urea denaturation experiments with characteristically pre-bound ADP at the D1-domain. Although a p97 hexamer is formed by six identical monomers, a fraction of the D1 sites is always pre-occupied by ADP, which is very difficult to release or exchange with other nucleotides. As a result, ATP in solution is only able to access the empty D1 sites, which drive the N-domain to the Up-conformation, whereas the N-domains remain in the Down-conformation for those subunits with pre-bound ADP. Although it is not yet clear how p97 maintains this asymmetry during its catalytic cycle, some level of communication must exist among subunits.

A model for the ATP cycle in the D1-domain and the corresponding N-domain conformation has been proposed by integrating the structural and biochemical data of wild type and mutant p97 (Fig. 6). The model proposes four nucleotide-binding states for the D1-domain. (1) There is the "ATP" state with ATP bound and the N-domain in the Up-conformation. Crystallographic and X-ray scattering experiments support the existence of this state in subunits of both mutant and wild type p97. It should be noted that due to non-exchangeable, pre-bound ADP in a wild type p97 hexamer, not all subunits can bring their N-domains to the Up-conformation, even with an excess of ATP in solution. (2) There is an "ADP-locked" state with non-exchangeable, pre-bound ADP at the D1 site and the N-domain in the Down-conformation. This state appears to be important for wild type p97 function and the pre-bound ADP is particularly difficult to release. The structure of the N-D1 fragment of wild type p97 may represent this conformation. (3) The "ADP-open" state is

defined by the binding of exchangeable ADP. This state was observed for mutant p97 by its biphasic ITC titration profile and is presumably in equilibration with the ADP-locked state. The structure of R155H with bound ADP may represent this conformation. (4) Finally, there is the “Empty” state with nucleotide-binding sites unoccupied and the N-domain in an unknown position. For wild type p97, the transition between the “ADP-locked” and “ADP-open” states is thought to be tightly controlled, resulting in rare ADP-open states, leading to asymmetry in the nucleotide binding and N-domain conformation in a hexameric p97.

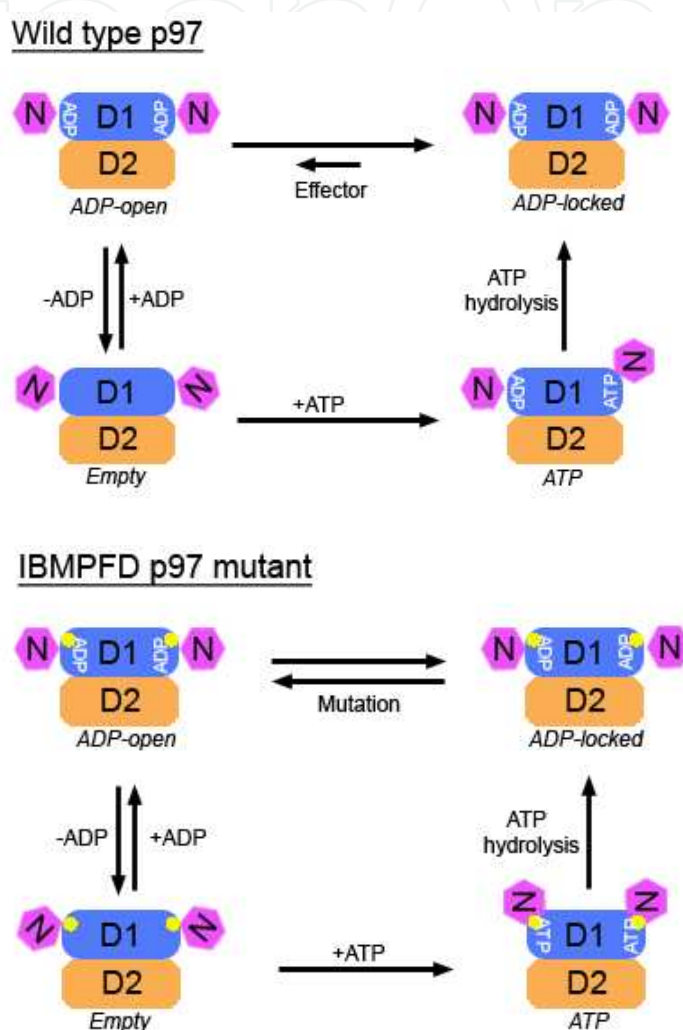


Fig. 6. **Models for the N-domain movement in p97 during ATP cycle** Schematic diagram for the control of the N-domain conformation in the wild type and IBMPFD associated N-D1 fragment of p97. Different domains are colored and labeled. The IBMPFD mutations are represented by yellow circles. Four states are defined for each nucleotide-binding site in D1: *Empty*, *ATP*, *ADP-locked* and *ADP-open* states, as labeled. The type of nucleotide bound at the D1-domain is labeled. Each subunit of the hexameric p97 is assumed to operate independently in this model.

From structural and molecular characterizations, we can infer that the non-uniform movement of p97 is essential to its function. In order to generate the up-and-down movement of the N-domain in a non-uniform fashion, the “ADP-locked” sites needed to be

activated to the “ADP-open” state. It is thought that in wild type p97 control of the transition between the ADP-locked and ADP-open state in D1 could be achieved in two ways: (1) the binding of adaptor proteins to the N-domain, or (2) the hydrolysis of ATP in the D2-domain. The N-domain was shown to have an influence on the ATPase activity of both N-D1 and the full-length p97 ortholog, VAT, as the N-domain-deleted p97 mutants have higher ATPase activity (Gerega et al., 2005). The binding of adaptor protein p47 to the N-domain was shown to inhibit the ATPase activity of p97 (Meyer et al., 1998). Communication between D1 and D2 is also known to exist for p97 and other type II AAA<sup>+</sup> proteins. For example, it was shown that the absence of D2-domain inhibits the nucleotide exchange activity in D1 (Davies et al., 2005). The yeast Hsp104, another type II AAA<sup>+</sup> protein, displays cooperative kinetics and inter-domain communication for its two ATPase domains (Hattendorf and Lindquist, 2002). However, the exact details of these possible control mechanisms for the switching of D1 nucleotide states remain elusive. Like many AAA<sup>+</sup> proteins involved in protein quality control such as *E. coli* ClpA and yeast Hsp104, p97 functions in handling protein substrates to various pathways, which requires the presence of the N-domain. Although how p97 handles these substrates has yet to be defined, one advantage of asymmetric interaction over symmetric seems that the former ensures continuous contacts with the substrates.

### 7.3 Loss of asymmetry in IBMPFD mutant p97

Mapping the IBMPFD mutations to the Down-conformation of p97 reveals the clustering of these mutations at the interface between the N- and D1-domains. Site-directed mutagenesis of R86A, a residue present at the N-D1 interface but not identified as an IBMPFD mutation, showed to possess all the structural and biochemical characteristics of an IBMPFD mutant p97 (Tang et al., 2010). This suggests that the N-D1 interface residues are critical for the proper function of p97 by providing tight regulation of the movement of the N-domain and the nucleotide state of the D1-domain.

Instead of prominent structural changes, IBMPFD mutations introduce subtle modifications to p97, apparently disrupting communication among the monomers. Unlike the wild type p97, IBMPFD mutants allow easy displacement of pre-bound ADP at the D1-domain by ATP $\gamma$ S, resulting in a unified nucleotide state, and hence, a symmetric hexamer in the Up-conformation. Using the same ATP catalytic cycle model for the D1-domain shown above, it was postulated that the difference between the wild type and mutants lies in the transition between the “ADP-locked” state and the “ADP-open” state. While this transition is tightly regulated in wild type p97, this control mechanism is altered in IBMPFD mutants, leading to a high concentration of subunits in the “ADP-open” state (Fig. 6). Consequently, p97 mutants undergo a uniform N-domain conformational change in response to high concentrations of ATP $\gamma$ S, leading to a defective enzyme.

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