The 97-kDa valosin-containing protein (p97-VCP) belongs to the AAA (ATPases associated with diverse cellular activities) family and acts as a molecular chaperone in diverse cellular events, including ubiquitin-proteasome-mediated degradation. We previously showed that VCP contains a substrate-binding domain, N, and two conserved ATPase domains, D1 and D2, of which D2 is responsible for the major enzyme activity. VCP has a barrel-like structure containing two stacked homo-hexameric rings made of the D1 and D2 domains, and this structure is essential for its biological functions. During ATPase cycles, VCP undergoes conformational changes that presumably apply tensions to the bound substrate, leading to the disassembly of protein complexes or unfolding of the substrate. How ATPase activity is coupled with the conformational changes in VCP complex and the D1 and D2 rings is not clear. In this report, we took biochemical approaches to study the structure of VCP in different nucleotide conditions to depict the conformational changes in the ATPase cycles. In contrast to many AAA chaperones that require ATP/ADP to form oligomers, both wild type VCP and ATP-binding site mutants can form hexamers without the addition of nucleotide. This nucleotide-independent hexamerization requires an intact D1 and the downstream linker sequence of VCP. Tryptophan fluorescence and trypsin digestion analyses showed that ATP/ADP binding induces dramatic conformational changes in VCP. These changes do not require the presence of an intact ATP-binding site in D1 and is thus mainly attributed to the D2 domain. We propose a model whereby D1, although undergoing minor conformational changes, remains as a relatively trypsin-resistant hexameric ring throughout the ATPase cycle, whereas D2 only does so when it binds to ATP or ADP. After ADP is released at the end of the ATP hydrolysis, D2 ring is destabilized and adopts a relatively flexible and open structure.

p97-VCP is a member of the highly conserved AAA (ATPases associated with diverse cellular activities) family (Ref. 1, and reviewed in Refs. 2–7) whose members are involved in a wide variety of cell functions. The family proteins are characterized by the presence of one (type I) or two (type II) highly conserved ATPase domains, also referred to as AAA domains. The ~250-amino acid ATPase domain contains the consensus Walker A and B motifs, which are responsible for ATP binding and ATP hydrolysis, respectively, and a second region of homology, which distinguishes the AAA family from other Walker-type ATPases. In addition to sequence homology, the family members almost always form a ring-shaped oligomeric structure. The contrast between the functional diversity and the high sequence and structural similarities suggests that AAA proteins play a common and fundamental role in cells, and the ring-shaped oligomeric structure is essential for such a role.

As other AAA proteins, VCP plays an important role in numerous seemingly unrelated cell activities, including membrane fusion (8–10), cell cycle regulation, stress response, programmed cell death, B and T cell activation, endoplasmic reticulum (ER)-associated degradation, and protein degradation (reviewed in Refs. 2–7). Interestingly, all these activities have been shown to be regulated, directly or indirectly, by the ubiquitin-proteasome degradation pathway. This notion suggests that VCP may play a fundamental role in the degradation pathway that underlies all these seemingly unrelated functions. Indeed, we and others (11–16) showed that VCP is genetically and functionally involved in the ubiquitin-proteasome degradation pathway. We further showed (11) that VCP physically associates with the ubiquitinated proteins, through a direct binding to the multiquitin chains, thus targeting the substrates to the proteasome for degradation. Recently, several groups (17–22) demonstrated that p97/VCP/Cdc48 along with its partners Ufd1 and Npl4 are required for ER-associated degradation. VCP likely acts as a molecular chaperone that extracts the ubiquitinated proteins from the ER membrane, modifies the conformation of the substrate proteins, and then targets the proteins to the 26 S proteasome for degradation. Moreover, VCP has also been proposed to work as a chaperone with cofactors, such as p47, SVIP, and VCP135, to mediate membrane fusion in Golgi, ER, and nuclear membrane assembly (23–25). The chaperone activity is powered by the energy generated from the ATP hydrolysis catalyzed by VCP. The conformational change of VCP during the ATPase cycle likely exerts mechanical force on the substrates and possibly the cofactors to accomplish the chaperone actions.

VCP molecule is composed of an N-terminal domain (N), two ATPase domains (D1 and D2), and a C-terminal domain (C). We previously showed that the entire VCP molecule is required for mediating the in vitro degradation of cyclin E (11). The N domain binds to the multiquitin chain and thus is responsible for substrate recognition (11), and both D1 and D2 are required for providing the chaperone activity (26). Whether the
two ATPase domains function equally and how they coordinate with each other is not understood. Previous reports have shown that the two ATPase domains of type II AAA proteins are different from each other with respect to sequence and function. Frequently, only one of them is a bona fide AAA domain, whereas the other exhibits lower similarity to the AAA consensus sequence. In NSF (27, 28) and Hsp104 (29), D1 is responsible for the major ATPase activity, whereas D2 mediates hexamerization. In bacterial ClpA and trypanosomatids TcPlB, the functions of the respective AAA domains are reversed (30–32).

Interestingly, D1 and D2 of VCP share high sequence similarities with each other and with those identified to mediate the ATPase activity. Thus, it is of particular interest to identify the specific functions of D1 and D2 in VCP. Indeed, we recently found that D1 and D2 are not enzymatically equal. Although D2 is responsible for the major ATPase activity at physiological temperature, D1 mediates a heat-enhanced ATPase activity (26).

Electron microscopy (EM) study indicated that VCP has a barrel-like homo-hexameric structure that comprises two-stacked hexameric rings made of the respective AAA modules (33). Although a crystallography study of an ADP-bound N-D1 domain provided significant structural details of the D1 ring (34), very little was known about the D2 ring and the conformation of respective rings during the ATPase cycle. We have taken biochemical approaches to characterize the conformation of VCP, either unbound or bound to ATP/ADP, with the goal of depicting the conformational changes accompanying ATP hydrolysis. We report that hexamer formation in VCP does not depend on the presence of nucleotide. An intact D1 and downstream linker region is required for the nucleotide-independent oligomerization. In addition, ATP/ADP binding induces dramatic conformational changes in D2. Both intrinsic Trp fluorescence and limited digestion studies suggest that D2 exhibits a relatively relaxed structure in the absence of nucleotide but forms a compact hexameric ring in the presence of ATP/ADP.

While this report was being prepared, Rouiller et al. (33) and Beuron et al. (35) reported cryo-EM studies that characterized the different conformations of VCP during the ATPase cycle. In general agreement with their findings, our study, using an independent, biochemical approach, provides additional molecular insights to the conformational changes of VCP during ATPase cycle.

MATERIALS AND METHODS

Mutagenesis and Protein Purification—ATP-binding site mutants, A1, A2, and A1A2, were generated as described previously (26, 36). The trypthophan to alanine substitutions were introduced into the Walker A motif of each or both of the ATPase domains, in which a highly conserved lysine was conserved to form a ring-shaped hexameric structure. Beuron et al. (35) reported cryo-EM studies that characterized the different conformations of VCP during the ATPase cycle. In general agreement with their findings, our study, using an independent, biochemical approach, provides additional molecular insights to the conformational changes of VCP during ATPase cycle.

Expression and Purification of Wild Type and Mutant VCP—To study the conformational and hexameric status of VCP at different nucleotide states (empty, ATP, or ADP), three groups of mutants were generated. Site-specific mutations were introduced into the Walker A motif of each or both of the ATPase domains, in which a highly conserved lysine was changed to threonine (Fig. 1A). Various deletion mutants were generated, into which progressive deletions from either the N or C terminus of VCP were introduced (see Fig. 3A). In addition, trypthophan at positions 454, 476, or 551 was mutated to alanine (Fig. 1A) for the fluorescence study. Wild type and mutant VCP were expressed as fusion proteins with a His tag fused at the C termini. All variants were subjected to consecutive affinity chromatography and purified to apparent homogeneity (Fig. 1B and 3B).

Hexamerization of VCP in the Absence of Nucleotide—Our previous study showed that VCP has an unusually high preponderance to form a ring-shaped hexameric structure. Because many AAA proteins assemble into oligomers in the presence of ATP but remain monomeric in the absence of nucleotide, we asked whether this is also true in VCP. Wild type VCP fusion protein was purified (Fig. 1B) and subjected to gel filtration chromatography in the absence of nucleotides. Remarkably, almost all VCP appears in a complex with a molecular size consistent with a hexamer (Fig. 1C). To further confirm this nucleotide independence, an A1A2 double mutant, which harbors a mutation in the ATP-binding site in both D1

Gel Filtration Chromatography—Affinity-purified His-tagged VCP and variants were centrifuged at 100,000 × g for 30 min before they were loaded onto a Superose 6 column (Amersham Biosciences) equilibrated with buffer containing 20 mM HEPES (pH 7.0), 150 mM NaCl, 4 mM MgCl2, 5 mM DTT, and 1% glycerol. Proteins were eluted at a flow rate of 0.5 ml/min and collected in 16 fractions (0.5 ml each). An aliquot of 20 μl from each fraction was analyzed by SDS-PAGE and immuno-blotting with antibody against the C terminus of VCP (15).

Fluorescence Spectroscopy—Fluorescence spectra of trypthphan were recorded with a PerkinElmer Life Sciences LS 50 luminescence spectrometer with the use of a 1.0-mm cell at approximately 24 °C. The excitation wavelength was set at 285 nm so as to monitor tryptophan emission between 305 and 450 nm with bandwidths of 5 nm. The protein concentration used was approximately 100 μg/ml. The experiments were carried out in the dialysis buffer plus 10 mM MgCl2 and either with or without 4 mM nucleotides.

Limited Proteolysis—Purified VCP (0.1 mg/ml) was incubated with 5 μg/ml trypsin (Roche Applied Science) at 37 °C for various periods of time in reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 1 mM DTT and with or without 4 mM ATP. The reactions were terminated by the addition of phenylmethylsulfonyl fluoride (0.5 mM) and SDS-gel sample buffer. The digestion products were separated into two parts. One part was analyzed by SDS-PAGE and stained with a SilverStain silver staining kit (Invitrogen). The other part was resolved on SDS-gel and immunoblotted with antiserum recognizing either the N or C terminus of VCP (15).

RESULTS

Expression and Purification of Wild Type and Mutant VCP—To study the conformation and hexameric status of VCP at different nucleotide states (empty, ATP, or ADP), three groups of mutants were generated. Site-specific mutations were introduced into the Walker A motif of each or both of the ATPase domains, in which a highly conserved lysine was changed to threonine (Fig. 1A). Various deletion mutants were generated, into which progressive deletions from either the N or C terminus of VCP were introduced (see Fig. 3A). In addition, tryptophan at positions 454, 476, or 551 was mutated to alanine (Fig. 1A) for the fluorescence study. Wild type and mutant VCP were expressed as fusion proteins with a His tag fused at the C termini. All variants were subjected to consecutive affinity chromatography and purified to apparent homogeneity (Figs. 1B and 3B).
The purified proteins (1/40 M VCP-His fusion proteins were expressed in E. coli WT fusion proteins. Wild type (WT) and mutant (as indicated on the top) VCP-His fusion proteins were expressed in E. coli and affinity-purified. The purified proteins (1 μg each) were resolved by SDS-PAGE and stained with Coomassie Blue. The molecular size standards are shown on the left. C, hexamer formation of wild type VCP in the absence of ATP. Purified wild type VCP-His fusion protein was subjected to Superose 6 gel filtration chromatography. The eluted fractions were analyzed by SDS-PAGE, Western transfer, and immunoblotting with anti-His antibody. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and albumin (66 kDa) were analyzed in parallel as standards. The same result was obtained for VCP prepared in the presence of ATP. A1A2-His fusion protein was analyzed as described in Materials and Methods.

Although the analyses were performed without the addition of nucleotides, it was formally possible that the purified recombinant VCP already contained a sufficient amount of nucleotides to maintain the hexameric structure. We thus boiled the VCP preparations to release the potentially bound nucleotide and then analyzed the sample by HPLC. The control experiment showed that ATP was not detected in VCP reassembled in the presence of ATP (not shown). D, hexamer formation of the A1A2 mutant. Affinity-purified A1A2-His fusion protein was analyzed as described in C.

Fig. 1. VCP fusion proteins are purified and analyzed for hexamerization. A, mutations at the ATP-binding sites and tryptophan residues. Lys (K) to Thr (T) and Trp (W) to Ala (A) mutations were introduced into the indicated ATP-binding sites, and tryptophan residues, respectively. B, affinity-purified wild type and mutant VCP-His fusion proteins. Wild type (WT) and mutant (as indicated on the top) VCP-His fusion proteins were expressed in E. coli and affinity-purified. The purified proteins (1 μg each) were resolved by SDS-PAGE and stained with Coomassie Blue. The molecular size standards are shown on the left. C, hexamer formation of wild type VCP in the absence of ATP. Purified wild type VCP-His fusion protein was subjected to Superose 6 gel filtration chromatography. The eluted fractions were analyzed by SDS-PAGE, Western transfer, and immunoblotting with anti-His antibody. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and albumin (66 kDa) were analyzed in parallel as standards. The same result was obtained for VCP prepared in the presence of ATP (data not shown). D, hexamer formation of the A1A2 mutant. Affinity-purified A1A2-His fusion protein was analyzed as described in C.

Fig. 2. ATP is detected in VCP reassembled in the presence of ATP, but not in purified wild type, A1A2, or VCP reassembled in the absence of ATP. A, retention time for ATP standard in HPLC analysis. ATP standard (200 μl of 2.5 μM) was analyzed by HPLC. The small peak eluted before ATP was also detected in the buffer control (not shown). B, no ATP in purified wild type and A1A2 VCP. Purified wild type and A1A2 samples (200 μl of 4 μM) were analyzed as described under “Materials and Methods.” C, detection of ATP in VCP reassembled in the presence of ATP. Wild type VCP (200 μl of 4 μM) was dissociated with 6 M urea and then reassembled in the presence or absence of ATP. The reassembled VCP was passed through Sephadex G-50 column to remove the unincorporated ATP and treated as described under “Materials and Methods” before the HPLC analysis. A similar result was obtained with ADP (not shown).

and D2 domains (Fig. 1A), was analyzed. The detection of A1A2 as hexamers (Fig. 1D) strongly supported that oligomerization of VCP does not require nucleotide binding.

Although the analyses were performed without the addition of nucleotides, it was formally possible that the purified recombinant VCP already contained a sufficient amount of nucleotides to maintain the hexameric structure. We thus boiled the VCP preparations to release the potentially bound nucleotide and then analyzed the sample by HPLC. Because the control ATP sample exhibits a sharp peak on the chromatogram (Fig. 2A), neither ATP (Fig. 2B) nor ADP (data not shown) was found in the purified wild type and A1A2 preparations. Furthermore, we dissociated VCP to monomers with 6 M urea, reassembled the dissociated VCP in the presence or absence of ATP/ADP (36), and then subjected the samples to HPLC analysis. As we previously reported, although hexameric VCP was efficiently reassembled in both conditions (36), ATP/ADP was only detected in the hexamers reassembled in the presence of added nucleotide (Fig. 2C). This result validated our ability to detect the ATP/ADP bound to hexameric VCP and further supported the nucleotide-independent nature of VCP oligomerization. Taken together, we conclude that nucleotide is not required for forming stable hexameric VCP complexes, an unusual property among AAA proteins.

D1-linker, but Not N or D2-C. Is Required for Nucleotide-independent Hexamerization—Next, we determined which one or more domains are responsible for the nucleotide-independent hexamerization. Prior crystallography study showed that the N-D1 domain (residues 2–458) of p97 forms a hexameric structure that tightly binds to ADP, and no nucleotide-free hexameric N-D1 was detected (34). This seems to suggest that N-D1 alone is not sufficient for the nucleotide-independent hexamerization. To identify the sequence requirement, we generated a series of deletion mutants with progressive N- or C-terminal truncations (Fig. 3A). The purified VCP variants (Fig. 3B) were subjected to native gel electrophoresis in the absence of ATP. As shown in Fig. 3C (also summarized in Fig. 3A), wild type and variants 1–511, 1–481, and 141–806 can form hexamers without the addition of nucleotides. These variants all contain an intact D1 (#200–458) and the subsequent linker (#458–473) region (designated as D1-linker) but lack at

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least part of the N, D2, or C domains. Thus, the D1-linker may be necessary for the hexamerization, and N, D2, and C are dispensable for the process. Moreover, variants 2–458, 443–806, and 268–806, all lacking at least part of the D1-linker region, failed to form hexamers (Fig. 3, A and C), indicating the requirement for D1-linker. Comparing variants 2–458 (N-D1) and 1–481, it is apparent that the linker region plays a critical role in nucleotide-free hexamerization. Without the linker, VCP is mostly present in monomeric form. This may provide the explanation why N-D1 cannot form hexamers without ADP as reported in previous crystallography study (34). We conclude that the D1-linker domain, but not the N or D2-C domain, is required for the nucleotide-independent hexamerization of VCP.

Intrinsic Tryptophan Fluorescence Study: Dramatic Conformational Change of D2 upon Nucleotide Binding—We previously demonstrated that the ATPase activity of VCP is required in carrying out its biological functions, such as ubiquitin-proteasome-mediated protein degradation. Thus, it is important to characterize the conformational changes of VCP during the ATPase reaction. Because a crystal structure of full-length VCP is not available, we took biochemical approaches to study the conformation of VCP either unbound or bound to ATP/ADP, representing specific phases of the ATPase cycle. Based on the facts that Trp fluorescence represents a sensitive probe for conformational change and wild type VCP contains three tryptophan (Trp) residues, we measured the changes in the fluorescence emission spectra of Trp under different nucleotide conditions. Because the experiments were carried out at room temperature, at which VCP exhibits negligible ATPase activity (26), the change in fluorescence after ATP addition is attributed to mainly the binding and not the hydrolysis of ATP. As shown in Fig. 4A and Table I, the Trp fluorescence of VCP peaks at a wavelength of 345 nm, and binding to ATP or ADP results in increases of 53.9% and 28.9% respectively. These increases suggest that nucleotide binding to VCP induces a conformational change in VCP molecule. The enhancement of fluorescence with ADP is weaker than that with ATP, probably implicating a less drastic conformational change. Therefore, VCP binding to ATP induces an initial conformational change, and further changes take place during the subsequent ATP hydrolysis and ADP release.

To determine whether D1 or D2 domain contributes to this ATP/ADP-enhanced Trp fluorescence, we further studied the ATP-binding site mutants, A1 and A2. Although the basal level and the peak wavelength (345 nm) of the Trp fluorescence of both mutants did not change from those of the wild type, the extent of fluorescence change in the presence of nucleotides varied significantly (Fig. 4B and Table I). When ATP and ADP were added, the Trp fluorescence of A1 increased 58.0% and 25.3%, respectively, similar to those observed in the wild type. By sharp contrast, the increase was totally abolished in A2. This result indicates that an intact ATP-binding site in D2, but not in D1, is necessary to bring about the ATP/ADP-enhanced Trp fluorescence of VCP. In other words, the Trp fluorescence change of VCP upon ATP/ADP binding reflects a significant conformational change in D2, but not in D1. Notably, this conformational change of D2 can take place without nucleotide binding to the D1 domain.
**Different D1 and D2 Ring Structure of p97-VCP**

### Fig. 4. Trp fluorescence of VCP is increased upon ATP and ADP binding to D2.

A. Trp fluorescence increase of WT VCP upon binding to ATP or ADP. The steady-state fluorescence spectra were measured in wild type VCP (0.1 mg/ml) in different nucleotide conditions (free, ATP, or ADP). B and C, requirement for an intact ATP-binding site in D2 for the nucleotide-induced fluorescence increase. ATP-binding site mutants, A1 (B) and A2 (C), were analyzed as described in A. The percentage of fluorescence increase in different nucleotide conditions is also summarized in Table I.

**Fluorescence Change in Trp-476 as the Indicator for Conformational Changes in D2**—To further characterize the ATP/ADP-increased Trp fluorescence, we identified the specific Trp residue that is responsible for the fluorescence changes. VCP contains three Trp residues at positions 454, 476, and 551. We changed each Trp to alanine to assess the impact (Fig. 1, A and B). Mutations of Trp-454 (Fig. 5A and Table I) and Trp-551 (Fig. 5B and Table I) did not affect the ATP/ADP-enhanced fluorescence pattern, although Trp-551 mutation resulted in a slightly lower basal level fluorescence. Thus, Trp-454 and Trp-551 are not the major contributors for the fluorescence changes in VCP upon ATP/ADP binding. Strikingly, the basal fluorescence intensity of W476A mutant was much lower than that of the wild type, and ATP/ADP binding did not enhance the fluorescence (Fig. 5C and Table I). The lack of fluorescence change is not a result of its incapacity to bind nucleotide or form hexamers, because the ATPase activity and hexamerization status of W476A mutant are the same as those of the wild type (data not shown). Therefore, Trp-476 is the main contributor to the ATP/ADP-enhanced fluorescence and serves as an indicator for the conformational change of D2. It should be noted that these data only assess the status of D2 and do not implicate a lack of changes in D1. It is possible that ATP/ADP binding also induces conformational changes in D1, but there is no appropriate Trp residue in D1 to indicate the changes, and/or the change in D1 is not sufficiently propagated to D2 to induce detectable fluorescence change in Trp-476.

**Protection of VCP from Trypsin Proteolysis in the Presence of Nucleotides**—To detect the conformational changes at a more global level, we then performed limited proteolysis in different nucleotide conditions. In this assay, it is presumed that a high sensitivity to proteolysis suggests a more open and dissociated conformation, whereas a low sensitivity indicates a relatively closed and associated conformation. Also, the efficiency of proteolytic cleavage at exposed sites on the surface of the complex correlates with the flexibility of the protein. The trypsin digestion pattern revealed by silver staining showed that VCP is readily digested in the absence of nucleotides after 40 min. However, in the presence of ATP, ADP, or ATP-γS an 87-kDa fragment (p87) is protected from digestion even after 100 min of incubation (Fig. 6A). Subsequent immunoblot analysis showed that p87 was only detected by the antisera specific to the N terminus (Fig. 6B) but not the C terminus (Fig. 6C) of VCP. Therefore, p87 consists of N, D1, and D2 domains (Fig. 6D), and the C-terminal the ~10–kDa fragment of VCP is readily cleaved off by trypsin and probably represents a loosely structured tail. It was also noted that, in the absence of nucleotide, several C-terminally truncated intermediates were observed (Fig. 6B). Among them, a major intermediate with a molecular size of ~58 kDa (p58) was detected at 60 min. Based on the calculated molecular size and similar gel mobility between p58 and VCP1–481 protein (data not shown), we deduced that p58 represents the N-D1-linker fragment (Fig. 6D).

These results suggest that, in the absence of nucleotide, the hexameric VCP complex comprises three substructures: a relatively compact and trypsin-resistant N-D1-linker structure, a more open and trypsin-sensitive D2, and a highly protease-sensitive C-terminal domain. In response to nucleotide binding, VCP undergoes a conformational change such that a trypsin-sensitive C-terminal domain. In response to nucleotide binding, VCP undergoes a conformational change such that a trypsin-sensitive C-terminal domain. Also, the efficiency of proteolytic cleavage at exposed sites on the surface of the complex correlates with the flexibility of the protein. The trypsin digestion pattern revealed by silver staining showed that VCP is readily digested in the absence of nucleotides after 40 min. However, in the presence of ATP, ADP, or ATP-γS an 87-kDa fragment (p87) is protected from digestion even after 100 min of incubation (Fig. 6A). Subsequent immunoblot analysis showed that p87 was only detected by the antisera specific to the N terminus (Fig. 6B) but not the C terminus (Fig. 6C) of VCP. Therefore, p87 consists of N, D1, and D2 domains (Fig. 6D), and the C-terminal the ~10–kDa fragment of VCP is readily cleaved off by trypsin and probably represents a loosely structured tail. It was also noted that, in the absence of nucleotide, several C-terminally truncated intermediates were observed (Fig. 6B). Among them, a major intermediate with a molecular size of ~58 kDa (p58) was detected at 60 min. Based on the calculated molecular size and similar gel mobility between p58 and VCP1–481 protein (data not shown), we deduced that p58 represents the N-D1-linker fragment (Fig. 6D).

These results suggest that, in the absence of nucleotide, the hexameric VCP complex comprises three substructures: a relatively compact and trypsin-resistant N-D1-linker structure, a more open and trypsin-sensitive D2, and a highly protease-sensitive C-terminal domain. In response to nucleotide binding, VCP undergoes a conformational change such that a trypsin-resistant N-D1-D2 hexameric structure is formed. This conformational change likely takes place more drastically in the D2 domain, which changes from a trypsin-sensitive to a resistant state.

**Requirement of Nucleotide Binding to D2 for Trypsin Resistance**—To demonstrate that ATP binding to D2 is responsible for the protection and stabilization of the D2 subcomplex, ATP-binding site mutants, A1 and A2, were subjected to limited trypsin proteolysis. As shown in Fig. 7A, when the binding site

| VCP variants | ATP   | ADP   |
|--------------|-------|-------|
| WT           | 53.9 ± 2.0 | 28.9 ± 1.7 |
| A1           | 58.0 ± 3.6 | 25.3 ± 2.8 |
| A2           | −0.25 ± 3.3 | −6.3 ± 3.7 |
| W454A        | 54.9 ± 6.3 | 27.1 ± 2.4 |
| W476A        | −8.7 ± 2.3 | 24.8 ± 5.6 |
| W551A        | 63.5 ± 10.2 | 36.6 ± 9.5 |

**TABLE I: Percent change of fluorescence of VCP variants upon ATP or ADP binding**

The mean ± S.D. changes in the peak fluorescence intensity of WT and mutant VCP are expressed relative to the nucleotide-free condition of each variant. The mean ± S.D. changes in the peak fluorescence intensity of WT and mutant VCP are expressed relative to the nucleotide-free condition of each variant.
in D1 is mutated ATP/ADP still protects the N-D1-D2 complex, resulting in the stabilization of p87 fragment as it does in the wild type (Fig. 6B). By contrast, mutation in D2 abolished this protection effect of ATP/ADP such that the predominant digestion product was the N-D1-linker fragment (p58) (Fig. 7A). This nucleotide-independent hexamerization domain in D1 is highly conserved in the P-loop and is the only Lys in the negatively charged residues in second region of homology of D1. Thus, an intact ATP-binding site in D2, but not D1, is required to confer protection of the N-D1-D2 complex from proteolysis. Several lines of evidence support that D1 is the major hexamerization domain in VCP: 1) the crystallography study of ADP-bound N-D1 reveals a hexameric ring structure (34); 2) we previously found that the ATP binding site in D1 plays an important role in the reassembly of urea-dissociated VCP (36); 3) our unpublished results indicate that changing the conserved Arg residues to negatively charged residues in second region of homology of D1 disrupts the hexamers; 4) the D1 domain is relatively resistant to protease digestion and likely stays as a trypsin-resistant ring throughout the ATPase cycle (Figs. 7B and 8); and 5) we identified the D1-linker as the major mediator for nucleotide-independent hexamerization (Fig. 3).

The difference in nucleotide requirement between VCP and other AAA hexamers may be explained by the structural details revealed by the crystallography studies of p97 N-D1 (34), NSF D2 (38, 39), and full-length ClpA (40). In the nucleotide-binding pocket of NSF D2, in addition to Lys-549 and Lys-708, another lysine, Lys-631, from one of the neighboring subunits also contacts the γ-phosphate of ATP (38, 39). Therefore, upon releasing ADP at the end of ATPase cycle, the resulting electrostatic repulsion between the three Lys residues and the change of hydrophobic interfaces lead to a dramatic structural destabilization. The recently solved crystal structure of full-length ClpA also reveals strong electrostatic interactions in D1, the major hexamerization domain. At least three positively charged residues from the neighboring subunit are within salt bridge distance from the bound ADP. In addition, five positively charged residues from the same neighbor also contribute to the nucleotide-binding site (40). Therefore, nucleotide binding is intimately coupled with hexamer formation in ClpA D1. By contrast, residues interacting with the β-phosphate of the bound ADP in p97 N-D1 are all in the vicinity of Lys-251, which is highly conserved in the P-loop and is the only Lys in the nucleotide binding pocket of p97 N-D1. Although two Arg residues (Arg-359 and Arg-362) from an adjacent protomer protrude into the nucleotide-binding pocket, Arg-362 forms a salt bridge with Glu-305 in the Walker B motif, leaving only Arg-359 to interact with the bound nucleotide (34). Because VCP lacks the electrical repulsion observed in NSF and ClpA, the hexamer would not be destabilized when ADP is released.

In this study, we report the novel finding that D1-linker upon Nucleotide Binding—A closer examination of Fig. 7B revealed that ATP/ADP binding to D1 does confer a slightly increased protection to the p58 N-D1-linker subcomplex. This observation suggests that nucleotide binding to D1 also induces conformational changes. To directly study this, we carried out trypsin digestion analysis on VCP 1–481 mutant (Fig. 3, A and B), which is devoid of the D2-C domain. As shown in Fig. 8, the presence of ATP or ADP indeed changes the protein to be more resistant to trypsin. However, the change is significantly less dramatic than that observed in D2 (compare Figs. 6B, 7A, and 8). In summary, VCP 1–481 exists as a stable hexamer in the absence of nucleotides, and the D1 ring undergoes minor conformational changes during the ATPase cycle.

DISCUSSION

Type II AAA chaperones, such as NSF, ClpA, ClpB, ClpY, and Hsp104, are present as monomers or dimers in the absence of nucleotides, but readily become hexamers in the presence of nucleotides. In comparison, VCP seems to have a much higher innate propensity to form hexamers (36). In this study, we show that hexamerization of wild type VCP does not require nucleotides (Figs. 1C and 2). This is further supported by the finding that ATP-binding site mutant, A1A2, also forms hexamers (Fig. 1D). This nucleotide-independent hexamerization is attributed to the D1-linker domain (Fig. 3), thus the D1 ring constitutively holds the hexamers together. Several lines of evidence support that D1 is the major hexamerization domain in VCP: 1) the crystallography study of ADP-bound N-D1 reveals a hexameric ring structure (34); 2) we previously found that the ATP binding site in D1 plays an important role in the reassembly of urea-dissociated VCP (36); 3) our unpublished results indicate that changing the conserved Arg residues to negatively charged residues in second region of homology of D1 disrupts the hexamers; 4) the D1 domain is relatively resistant to protease digestion and likely stays as a trypsin-resistant ring throughout the ATPase cycle (Figs. 7B and 8); and 5) we identified the D1-linker as the major mediator for nucleotide-independent hexamerization (Fig. 3).

In D1 is mutated ATP/ADP still protects the N-D1-D2 complex, resulting in the stabilization of p87 fragment as it does in the wild type (Fig. 6B). By contrast, mutation in D2 abolished this protection effect of ATP/ADP such that the predominant digestion product was the N-D1-linker fragment (p58) (Fig. 7B). Thus, an intact ATP-binding site in D2, but not D1, is required to confer protection of the N-D1-D2 complex from proteolysis. In other words, D1 almost always forms a trypsin-resistant ring regardless of the nucleotide conditions, but D2 only does so when it binds to nucleotides.

FIG. 5. Trp-476 is a critical indicator for the fluorescence change. A and B, no impact in fluorescence spectra in W454A and W551A mutants. The steady-state fluorescence spectra were measured in W454A and W551A mutants (0.1 mg/ml) in different nucleotide conditions (free, ATP, or ADP). C, requirement of Trp-476 for ATP/ADP-induced Trp fluorescence increase. The steady-state fluorescence spectra were measured in W476A mutant (0.1 mg/ml) were determined in different nucleotide conditions (free, ATP, or ADP). The percentage of fluorescence change in W476A mutant (0.1 mg/ml) were determined in different nucleotide conditions (free, ATP, or ADP).
sequences are required for the nucleotide-independent hexamerization. The absolute requirement for the linker region in this event explains why N-D1 could not form hexamers on its own (34). The high trypsin resistance of the p58 N-D1-linker (containing 18 and 1 trypsin cleavage sites in D1 and linker, respectively) suggests that the linker region is sterically inaccessible to the protease. Moreover, our unpublished result also suggests that this linker region is at least partially buried. This is because when a PreScission protease (Amersham Biosciences) cleavage site was inserted between residues 459 and 460, whereas the protease readily cuts the urea-dissociated VCP, it could not access the hexamer at any nucleotide states (empty, ATP, or ADP). Because the linker sequence was originally defined by sequence alignment, it is possible that this linker region (or at least part of it) could be considered part of the D1 domain.

The Trp fluorescence study showed that VCP binding to ATP and ADP induces a fluorescence increase of −54 and −29%, respectively (Fig. 4 and Table I). Site-specific mutant analyses further indicated that this fluorescence change is mainly contributed by nucleotide binding to the D2 domain. Although VCP molecule has three Trp residues (positions 454, 476, and 551), only Trp-476 is critical for this ATP/ADP-induced fluorescence change. Thus Trp-476 can serve as the indicator for the fluorescence assay (Fig. 5 and Table I). The change is unlikely resulted from a direct contact between Trp-476 and the nucleotide, because W476A mutant still binds ATP and maintains a wild type ATPase activity (data not shown), and moreover, functional alignment of D1 and D2 does not suggest a direct interaction between Trp-476 and nucleotide. Thus, the Trp fluorescence increase likely results from the conformational changes of VCP induced by nucleotide binding to D2 domain without direct contacting Trp-476. Our unpublished result also showed that VCP variant that contains a PreScission protease cleavage site inserted at position 476 could only be cut in the presence of ATP, which obviously changes the peptide segment...
from a buried to an exposed state. Hence, ATP binding to D2 domain probably induces intra- and inter-subunit conformational changes, resulting in alterations in the microenvironment around the Trp-476 residue. It should be noted that, because D1 domain does not have an indicator Trp residue, this assay could not be used to assess the possible conformational changes in the D1 domain.

Limited trypsin digestion has been used to characterize the conformational changes resulted from oligomerization of a number of AAA proteins, e.g. NSF (41). Comparison of the proteolysis patterns of the wild type, A1, A2, and VCP 1–481 in different nucleotide conditions (Figs. 6–8) led to the conclusions consistent with those obtained in fluorescence study. Although N-D1-linker subcomplex has a relatively compact and trypsin-resistant structure, it exhibits minor conformational changes during the ATPase cycle. On the other hand, the D2 subcomplex is much more flexible. In the context of a full-length VCP hexamer, D2 converts from a trypsin-sensitive to a resistant form after ATP/ADP binding. Therefore, in the presence of nucleotide, N-D1-D2 (p87) is still protected from trypsin digestion even after a prolonged reaction (>60 min) (Fig. 6). These data directly suggest that during the ATPase cycle D2 forms a compact hexamer upon ATP binding but destabilizes and adopts a more relaxed and open structure after ADP release. This result is consistent with our previous finding that at physiological temperature D1 exhibits very low ATPase activity but D2 mediates the major enzyme activity. It is speculated that at physiological temperature the two AAA domains carry out distinct functions during the ATPase cycle. The D1 ring, although it also undergoes minor conformational changes, provides a structural framework that keeps the substrate-binding N domain and the enzymatic D2 domain in a proper context. On the other hand, the primary function of D2 is to carry out ATP hydrolysis, which couples with the conformational changes that can be propagated to the D1 and N domains. Our previous study showed that, at elevated temperature, e.g. 50 °C, D1 is capable of mediating heat-enhanced ATPase activity. It is possible that at such an elevated temperature the D1 ring is expanded and less rigid, and becomes more D2 like, in terms of undergoing more dramatic conformational changes and catalyzing ATP hydrolysis.

While the manuscript for this report was being prepared, Rouiller et al. (33) and Beuron et al. (35) reported cryo-EM studies of VCP at different nucleotide conditions. Based on the molecular “snapshots” captured in these studies, models were proposed to depict the conformational changes of VCP during the ATPase cycle. Interestingly, although in general agreement, the two studies report a number of conflicting findings.
For instance, Rouiller et al., but not Beuron et al., showed six side protrusions associated with the D2 ring near the central plane of the p97 hexamer (33). Although the identification of this side protrusion is not clear, two possibilities have been proposed, namely the linker region (residues 458–473) between D1 and D2 and the C-terminal tail (33). The side protrusions appear on the surface of the hexameric complex and are expected to be accessible to proteases. Nevertheless, our trypsin and PreScission digestion experiments showed that the linker region is inaccessible to the proteases and at least partially buried (see above). Thus, the linker is not likely to be the side protrusion detected on the surface of the complex. On the other hand, our limited proteolysis study (Figs. 6 and 7A) indicated that the C-terminal tail of VCP (approximately from residue 733 to 806) is readily digested. Given the fact that this region contains five trypsin cutting sites and that trypsin digestion failed to recover a ~10-kDa C-terminal fragment, the C domain likely is loosely structured and hence readily cleaved. Together, our results would favor the speculation of C domain being the side protrusion.

In general, our findings are in close agreement with those reported by Rouiller et al., in the following aspects: 1) hexamer formation does not require the presence of nucleotide; 2) the N domain is not required for hexamerization; 3) D1 undergoes minor conformational changes during ATP hydrolysis; and 4) nucleotide binding induces pronounced conformational changes in D2. Although our proteolysis assays did not detect significant differences between the ATP- and ADP-bound forms of VCP, Trp fluorescence analysis revealed distinct profiles of the two states. Our previous study showed that ATP-bound VCP can bind polyubiquitin chains, but ADP form cannot. Because N domain is responsible for binding to the substrate and cofactors, the ADP-induced conformational change must be sufficient to cause the N domain to release the ligand. Presumably, N domain also undergoes coordinated conformational or positional changes to bind or to release the ligand. In agreement, both EM studies demonstrated that N domain is highly mobile and undergoes significant positional changes during the ATPase cycle (33, 35). Crystallography study identified a partially buried linker region (residues 186–208) between the N and D1 domains (34). This linker supposedly works as an arm that moves the N domain up and down or around the periphery of D1 ring. Based on the high flexibility of this “linker arm,” it is predicted to be readily accessible to proteases. Unexpectedly, despite the presence of two potential cleavage sites in this linker sequence (Lys-190 and Arg-191), this region is not readily digested by trypsin. We speculate that, whereas the majority of the linker may be indeed exposed and mobile, the sequences around the trypsin cleavage sites may happen to be buried in the complex. Hence, our inability to detect ready digestion in this region is because of the lacking of trypsin sites in the exposed and mobile region rather than physical inaccessibility.

Based on the biochemical data presented in this study, we propose the following model (Fig. 9). The full-length VCP exists in a homo-hexameric structure regardless of the nucleotide condition. Neighboring D1-linker domains form a relatively stable ring, which holds the full-length VCP hexamer together throughout the ATPase cycles. Although D1 undergoes detectable conformational changes during the ATPase cycle, the changes are relatively minor. On the other hand, the hexameric ring made of the neighboring D2 domains is much more mobile. It is more open and relaxed in the absence of nucleotide, but adopts a more closed and compact conformation after binding to nucleotide. Although the differences between the ATP and ADP forms of VCP is not specified in this study, the ADP-bound VCP adopts a unique conformation (illustrated by strips) such that it is incapable of binding to the substrate (11). Obviously, this model needs to be further tested by other experimental systems, such as high resolution structures in different nucleotide conditions.

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