研究工作詳述：

The overall goal of this research project is to deliver crystal structures of human antizyme (Az) in complexes with its various target proteins, including ornithine decarboxylase (ODC), antizyme inhibitor (AzI), cyclin D1, and Aurora A kinase. These structures are expected to unravel the molecular basis by which Az recognizes its binding partners. In addition, we will use an N-terminal truncated version of Az (Az_{95-228}), which possesses ODC-binding activity and are capable of facilitating the association between ODC and the 26S proteasome but WITHOUT promoting the subsequent proteolytic destruction of ODC, to study how Az-binding allows its targets to be recognized by the proteasome. This work is expected to bring significant new mechanistic insights regarding the Az-mediated protein degradation pathway. **Preliminary results directly related to the proposed studies are summarized in Sections I-I, I-II, and I-III**.

The main research interest of my lab is to understand the structure/function relationship of protein molecules by using X-ray crystallography as the main tool. By applying this powerful technique, we have determined and deposited many high-resolution protein structures over the past few years (PDBid: 3L6V; 3B6H; 3B98; 3B99; 2IAG; 2GSC; 1WP5; 2D27; 2D28; 1RPS; 1RQ3; 1RQ4; 1RQA; 3QX3; 3QZD; 3QYV; 3QZK), including the structures of human type II topoisomerases in complexes with DNA and anticancer drugs (one paper published in *Science*, two papers are currently being prepared for publication), the C-terminal domains of bacterial type II topoisomerases (papers published in *Journal of Biological Chemistry* and *Nucleic Acids Research*), human prostacyclin synthase and its complexes with substrate analog and inhibitor (papers published in *Journal of Biological Chemistry* and *Journal of Molecular Biology*), the conserved hypothetical protein Xcc441 from *Xanthomonas campestris* (paper published in *Proteins*), the N-terminal domain of secretion ATPase XpsE (paper published in *Journal of Biological Chemistry*). It is fair to conclude that my lab has established the ability to perform all contemporary techniques and methodologies in the field of macromolecular crystallography. **Selected results are summarized in Sections I-IV, I-V, and I-VI**.

Preliminary results directly related to the current proposal:

I-I. Structural Study of Human Ornithine Decarboxylase in Complex with the C-terminal Domain of Antizyme

Polyamines, including spermidine, spermine, and putrescine, are positively charged small organic cations. By interacting with negatively charged nucleic acids and acidic surface patches of proteins, these compounds are involved in a large number of cellular processes, ranging from functional modulations of ion channels, nucleic acid packaging, DNA replication, to transcription and translation. Therefore, polyamines are essential for cell growth and differentiation, and aberrant cellular polyamine level has been implicated in neoplastic transformation. L-Ornithine decarboxylase (ODC) catalyzes the first and rate-limiting step in the polyamine biosynthetic pathway, and its enzymatic activity is subjected to a tight regulation. In mammals, ODC is targeted for proteasomal degradation by interacting with

antizyme (Az), a 26.5 kDa intracellular protein that binds ODC to form a non-covalent 1:1 complex. Az-binding induces a conformational change at the ODC C-terminal region, which triggers degradation via the 26S proteasome in an unique ubiquitin-independent manner. To decipher how Az recognizes ODC and how Az-binding promotes proteasomal proteolysis of ODC, we have obtained a crystal structure of ODC in complex with the C-terminal domain of Az (AzΔN; residues 95~228) (Fig. 1), which displays similar ODC-binding affinity as the full-length Az. The substantial overlap between the Az-binding surface and the homodimerization interface of ODC readily explains why the formation of a catalytically active ODC dimer is blocked in the presence of Az (Fig. 2). Moreover, a proposed proteasome- targeting region of ODC undergo conformational changes and become surface-exposed upon Az binding, likely allows its recognition by the 26S proteasome (Figs. 3 and 4). This work also explains the functional divergence of Az isoform.

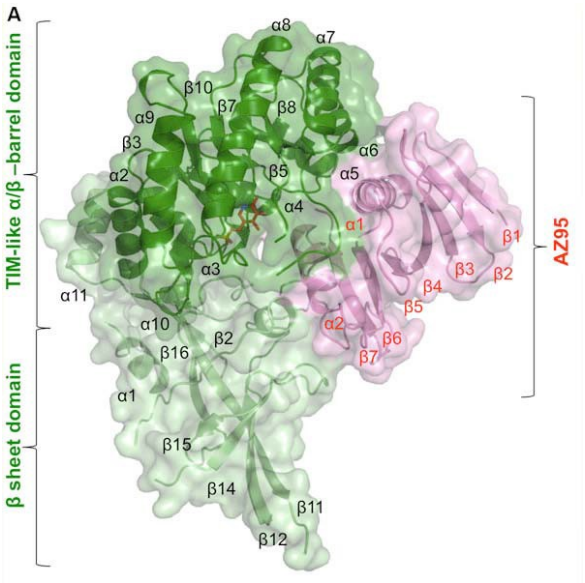
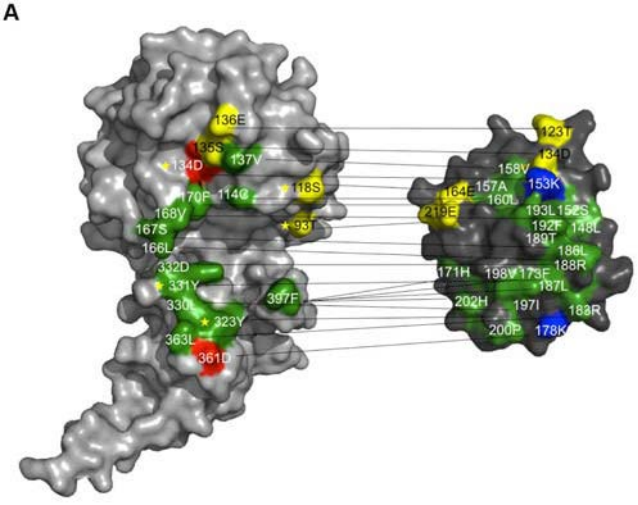


Fig. 1. Overall structure of the ODC-AzΔN complex.
The ODC TIM-like α/β-barrel domain is shown in forest green, the β sheet domain in pale green, and the AzΔN in pink.

Fig. 2. Interaction between ODC and AzΔN.
(**A**) Surface view showing exposed residues involved in ODC-AzΔN interaction. Interacting residues in green (hydrophobic interaction), yellow (hydrogen bond formation), in red and blue (salt bridge formation). (**B**) List of interacting residues revealed by the ODC-AzΔN complex. The asterisk (*) denotes those residues involved in both ODC homodimerization and ODC-Az heterodimerization.

A



B

hODC	AZ	hODC	AZ	hODC	AZ
*T93	E219	S167	T189	*L330	I197
C114	A157	V168	L148	*Y331	L187
C114	L160	V168	S152	D332	R188
*S118	E164	V168	K153	*D361	K178
*D134	K153	V168	T189	L363	K178
S135	D134	F170	K153	L363	R183
E136	T123	F170	L193	*F397	H171
V137	V158	*Y323	I197	*F397	F173
L166	R188	*Y323	P200	*F397	V198
L166	F192	*L330	L187	*F397	H202
S167	L186				

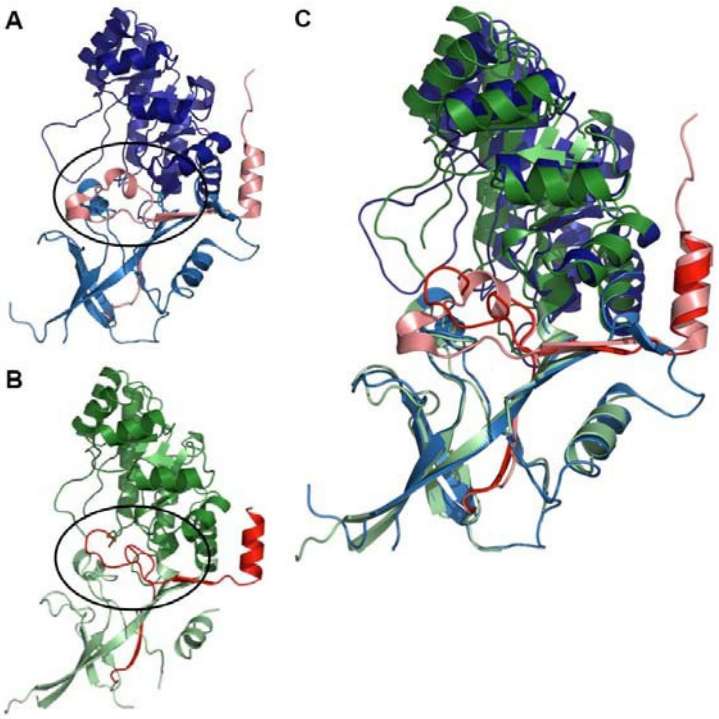


Fig. 3. The Az-induced tertiary structure changes of ODC C-terminal region.
(A-C) Significant conformational differences between ODC homodimer and ODC-Az95 heterodimer structure lie in the ODC C-termianl region.

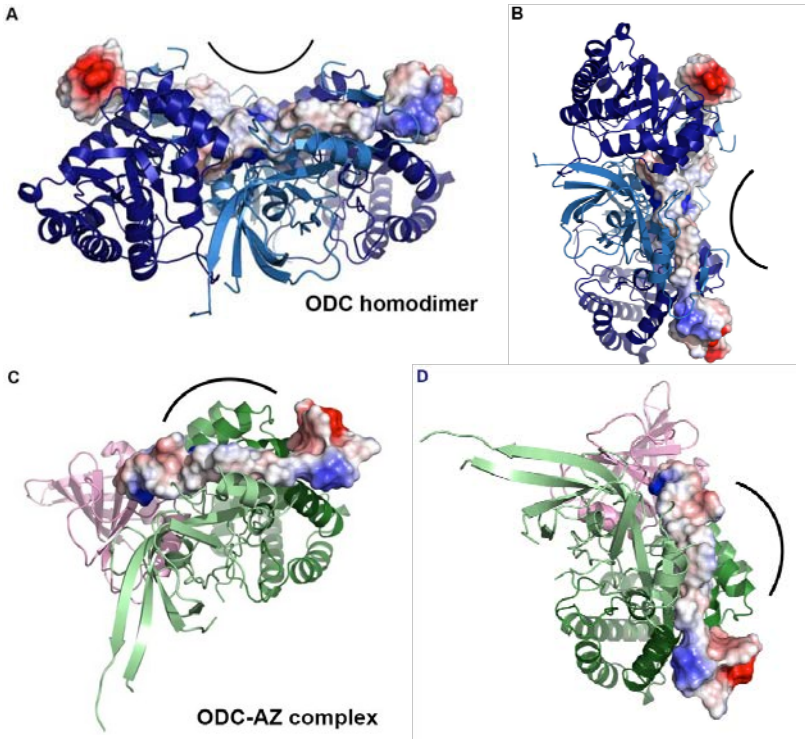


Fig. 4. Az-binding promotes proteasomal proteolysis of ODC. (**A** and **B**) Electrostatic surface potential of the C-terminal fragments of ODC homodimer. (positive potential, blue; negative potential, red). (**C** and **D**) The electrostatic surface shows that Az-binding induces prominent conformation changes in the ODC C terminal region (residues 393-421). As a result, the previously partially buried proteasome-binding fragment in the ODC homodimer interface becomes fully surface-exposed.

I-II. Structural Analysis of Human Antizyme in Complex with Antizyme Inhibitor

Polyamines are a group of low molecular weight organic polycations abundantly present in cells. Through their interactions with negatively charged nucleic acids and acidic surface patches of proteins, these compounds can participate in a large number of cellular processes, ranging from maintenance of DNA structure, replication, RNA processing, to translation and protein activation. Hence, polyamines are indispensable for cell growth and differentiation, and aberrant cellular levels of which are known to be associated with many diseases. ODC catalyzes the first reaction in the polyamine biosynthesis pathway and its enzymatic activity is subjected to tight

regulation. In mammals, elevation of cellular polyamine concentration induces the expression of Az, an intracellular protein with ODC-binding activity. Formation of the Az-ODC heterodimer not only inhibits the enzymatic activity of ODC, but also triggers ODC degradation via the 26S proteasome in an unique ubiquitin-independent manner. Besides the Az-mediated negative regulation, the intracellular polyamine homeostasis is also controlled by an enzymatically inactive ODC homolog termed antizyme inhibitor (AzI), whose higher affinity for Az may interfere with Az-mediated degradation of ODC and increase cellular polyamine level. Unlike ODC, the half-life of AzI increases upon Az binding, possibly by preventing the polyubiquitination of AzI. To understand how Az interacts with AzI and why the binding of Az stabilizes AzI, we have obtained a crystal structure of AzI in complex with the C-terminal domain of Az (AzΔN; residues 110-228) (Figs. 5 and 6). While Az triggers large structural rearrangement in ODC, no significantly AzI conformational change was induced upon its association with Az, suggesting a “key-and-lock” type of binding between Az and AzI (Fig. 7). Our structure also reveals two α helixes of Az as key AzI-binding elements (Fig. 8). We speculated that Az and an E3 ubiquitin ligase may compete for the same binding region on AzI, such that polyubiquitination of AzI is inhibited in the presence of Az.

Fig. 5. Experimental electron density map of AzΔN. Although the AzΔN-AzI diffraction data set was collected at low resolution (~5.8 Å), features of the extra piece of electron density (enclosed in white circle) are consistent with the presence of AzΔN.

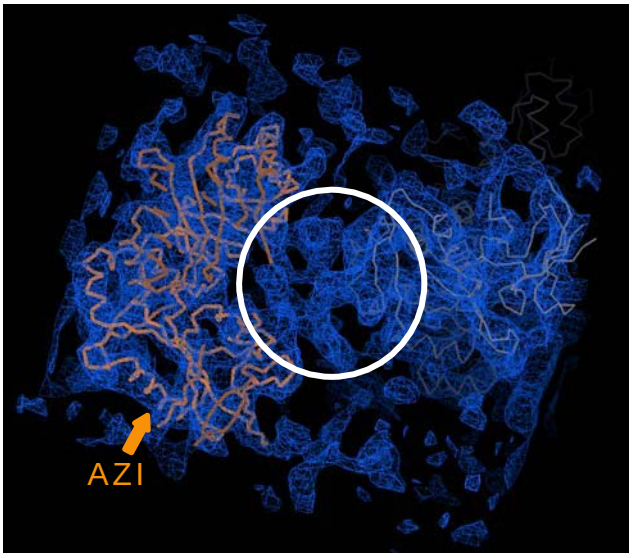
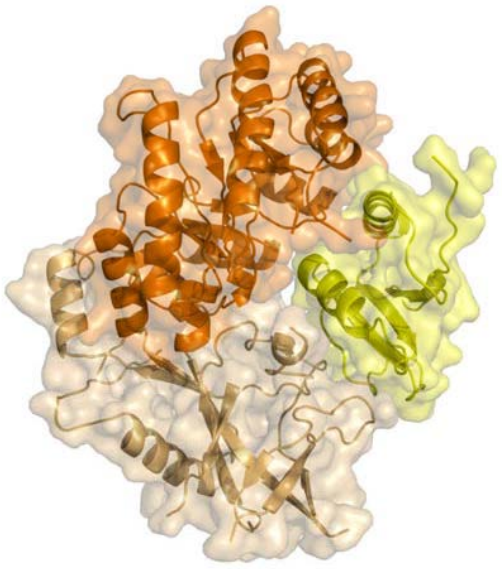


Fig. 6. Overall structure and crystallographic parameters of AzΔN-AzI. The AzI TIM-like α/β-barrel domain is shown in dark brown, the β sheet domain in light brown, and the AzΔN in yellow.



	AZIAZ110
Resolution range (Å…)	25.92 - 5.812
Space group	*P* 32
Unit cell	166.563 166.563 144.864 90 90 120
Total reflections	
Unique reflections	12060
Multiplicity	
Completeness (%)[a]	98.46
I/sigma(I)	11.00(1.83)
Wilson B-factor	360.38
R-sym[b]	
R-factor[c]	0.1911
R-free[c]	0.2499
Number of atoms	7460
Protein residues	967
Water molecules	
RMS(bonds)	0.003
RMS(angles)	0.81
Ramachandran [d] favored (%)	97
Ramachandran outliers (%)	0
Clashscore	7.00
Average B-factor	84.90

Fig. 7. Superposition of AzΔN-ODC and AzΔN-AZI complex. Unlike ODC which exhibits significant Az-induced structural changes, no significantly conformational changes of AzI was observed upon its association with AzΔN, suggesting a "key-and-lock" type of binding between the two.

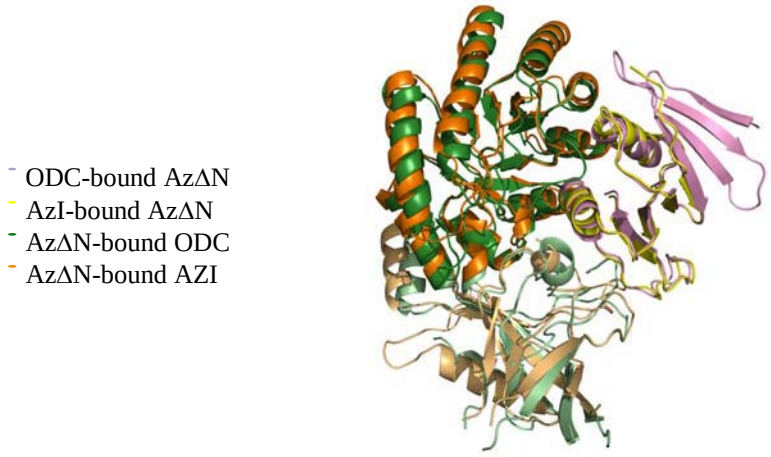
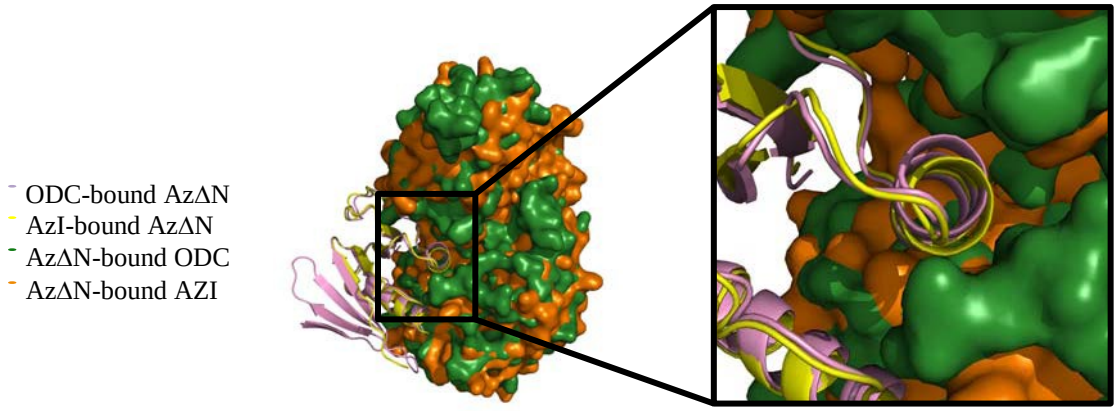


Fig. 8. Two α helixes of Az may serve as key AzI-binding elements.



I-III. Functional Verification of Residues Involved in ODC-Az Binding (This work was carried out via collaboration with Prof. Hui-Chih Hung, co-PI of this grant proposal.)

It has been well established that the homodimeric form of ODC is disrupted upon the formation of ODC-Az heterodimer. To verify those interacting residue pairs observed in the ODC-AzΔN crystal structure are functionally relevant, size distributions of wild-type and mutant forms of ODC in the presence of different Az concentration were analyzed (Fig. 9). All sedimentation data were globally fitted with the AB hetero-association model in the SEDPHAT program to obtain the dissociation constant (K_d) between ODC and Az (Table 1). As expected, Az_wt interrupted the dimers of ODC_wt to form Az-ODC heterodimer with a $K_{d,ODC\text{-}Az}$ of 0.71 μM (Table 1). We then mutated six residues present in the interface of ODC-AzΔN complex to create a series of single mutants of ODC: ODC_S118A, ODC_D134A, ODC_S135A, ODC_Y331A, ODC_D361A and ODC_F397A. The ODC_S135A, ODC_Y331A, and ODC_F397A interact with Az (Fig. 9) with $K_{d,ODC\text{-}AZ}$ values of 2.4, 3.2 and 1.9 μM, respectively (3.4, 4.5 and 2.7-fold higher than that of ODC_wt, Table 1). Furthermore, the ODC_S118A, ODC_D134A, and ODC_D361A mutants showed much weaker binding toward Az (Fig. 9) with $K_{d,ODC\text{-}AZ}$ values of 5.3, 8.1 and 13.9 μM, respectively (7.5, 11.4 and 19.6-fold higher than that of ODC_wt) suggesting their importance for Az-binding.

In the structure of ODC-AzΔN complex, ODC-Ser118, ODC-Asp134, and ODC-Asp361 may be ion-paired or hydrogen-bound with Az-Glu164, Az-Lys153, and Az-Lys178, respectively, and these polar interactions may be the critical factors governing the Az-binding affinities between human ODC and Az. Thus in a reciprocal study, we mutated these three residues in Az: Az_K153A, Az_E164A, and Az_K178A. These three mutants interact with ODC (Fig. 10) with $K_{d,ODC\text{-}Az}$ values of 1.2, 1.7 and 4.2 μM, respectively (1.7, 2.4 and 5.9-fold higher than that of Az_wt, Table 1). Since the single mutants Az_K153A and Az_E164A showed similar binding affinity toward ODC, we produced a double mutant Az_K153A/E164A and examined its ability to interact with ODC (Fig. 10). The $K_{d,ODC\text{-}Az}$ value of this double mutant was about 5.3 μM (Table 1) suggesting a synergistic effect between Az-Glu164 and Az-Lys153 for stable binding toward ODC. We also examined the Az-binding affinity of the double mutant ODC_S118A/D134A in which the polar contacts with Az-Glu164 and Az-Lys153 may be disrupted (Fig. 9). Taken together, our mutagenesis studies provide additional supports for the functional relevance of the ODC-AzΔN structure.

Fig. 9. Continuous sedimentation coefficient distribution of human wild-type and mutant ODC in the presence of Az. The concentration of ODC was fixed at 0.3 mg/mL with concentrations of Az ranging from 0.03 to 0.2 mg/mL (the molar ratio of Az/ODC ranged from 0.25 to 2) in 30 mM Tris-HCl (pH 7.4) at 20 °C. The sedimentation velocity data were globally fitted with the SEDTHAT program to obtain K_d values for the AZ -ODC complex (Table 1).

Figure W

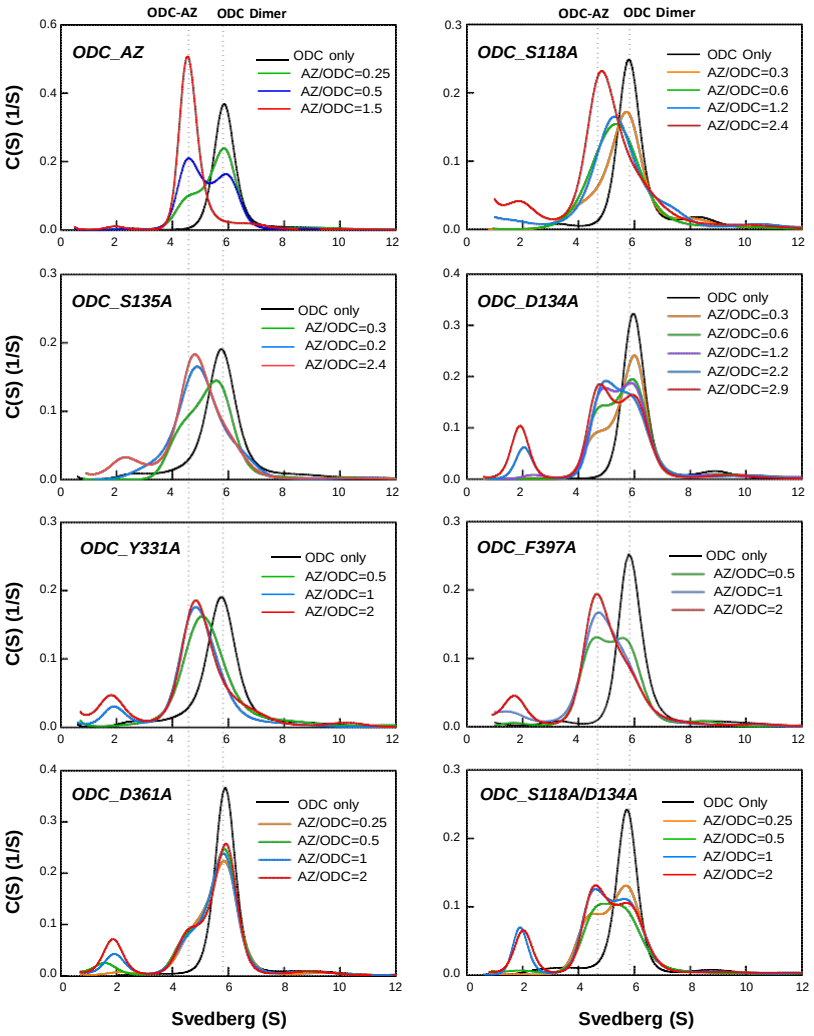


Fig. 10. Continuous sedimentation coefficient distribution of human ODC in the presence of wild-type and mutant Az. The concentration of ODC was fixed at 0.3 mg/mL with concentrations of Az ranging from 0.03 to 0.2 mg/mL (the molar ratio of Az/ODC ranged from 0.25 to 2) in 30 mM Tris-HCl (pH 7.4) at 20 °C. The sedimentation velocity data were globally fitted with the SEDTHAT program to obtain K_d values for the Az -ODC complex (Table 1).

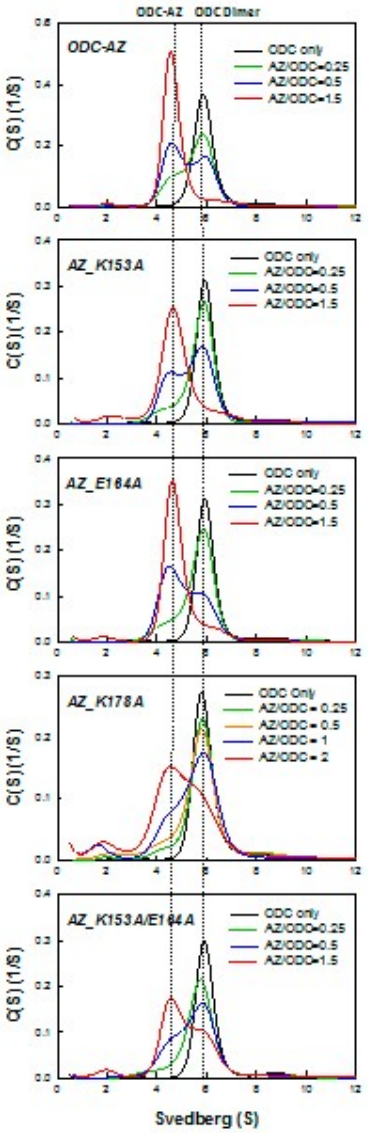


Table 1 Dissociation constants of human ODC-AZ complexes

	$K_{d,AZ-ODC}$ (μM)		$K_{d,AZ-ODC}$ (μM)
ODC-AZ	0.7 ± 0.01		
[ODC_S118A]-AZ	5.3 ± 0.04	ODC-[AZ_K153A]	1.2 ± 0.01
[ODC_D134A]-AZ	8.1 ± 0.06	ODC-[AZ_E164A]	1.7 ± 0.01
[ODC_K135A]-AZ	2.4 ± 0.02	ODC-[AZ_K178A]	4.2 ± 0.04
[ODC_Y331A]-AZ	3.2 ± 0.03	ODC-[AZ_K153A/E164A]	5.3 ± 0.04
[ODC_D361A]-AZ	13.9 ± 0.23		
[ODC_F397A]-AZ	1.9 ± 0.02		
[ODC_S118A/D134A]-AZ	8.2 ± 0.07		

The dissociation constants (K_d) of Az-ODC were derived from global fitting of the sedimentation velocity data to the model of A+B↔AB hetero-association in the SEDTHAT program.

II-I. Uniqueness and significance of this proposal

The selective removal of cellular proteins by the 26S proteasomal protein degradation pathway controls the activities of numerous biological processes (Schrader *et al*., 2009; Finley 2009), including cell cycle progression, regulation of gene expression, stress responses, apoptosis, and cell differentiation and reprograming. Two labelling systems have been identified for the hihgly specific tagging of those unneeded or damaged proteins for proteasomal degradation, one via the covalent incorporation of poly-ubiquitin chain(s) onto the target proteins, the other involves noncovalent association between the target proteins and Az (Kahana 2009). Compared to the ubiquitin-dependent tagging system, where the structural basis underlying substrate protein recognition by various ubiquitin E3 ligases has been studied extensively, surprisingly little is known regarding the specificity determinants of Az-mediated degradation pathway. While the crystal structures of two Az-interacting proteins, ODC and AzI (Almrud *et al.,* 2000; Albeck *et al*., 2008), as well as the solution (NMR) structure of an Az fragment had been determined (Hoffman *et al*., 2005), no experimentally determined structures of the ODC-Az and AzI-Az complexes are available. **As described in C012-1, we have successfully obtained crystals and preliminary structural information of both Az-ODC and Az-AzI, and are now in a great position to deliver crystal structures of additional complexes formed by Az and its target proteins.** Moreover, using the unique property of $Az_{95\text{-}228}$, which can target ODC to the 26S proteasome but without promoting the subsequent proteolytic destruction of ODC, a strategy has been proposed to examine how Az-binding allows its targets to be recognized by the proteasome. These new structures and results are expected to provide new mechanistic insights for the Az-mediated protein degradation pathway by addressing the following outstanding questions:

(1) How does Az recognize its target proteins?
(2) How does Az-binding allow ODC to be recognized by the 26S proteasome?
(3) How does Az interfere with the homodimerization of ODC?
(4) Does Az-binding induce functionally significant conformational changes in ODC?
(5) Why the other Az isoforms (Az2, Az3, Az4) fail to promote proteasomal degradation of ODC?
(6) Why Az exhibits higher affinity for AzI than ODC, despite the high degree of similarity between the two?
(7) Why is AzI not targeted for degradation upon Az-binding?
(8) Can small molecule compounds with potential anticancer activity be designed to bind and trigger conformational changes in ODC to promote its destruction?
(9) Where is the docking site/subunit of 26S proteasome that interacts directly with ODC-Az?

II-II. International competitiveness

1. The labs of Chaim Kahana (Weizmann Institute of Science, Israel) and Marvin Hackert (University of Texas) are the leading players in this field: the Kahana lab has recently solved the crystal structure of AzI (Albeck *et al*., 2008); Hackert lab published the NMR structure of Az C-terminal domain (Hoffman *et al*., 2005) and the crystal structure of ODC (Almrud *et al.*, 2000). Up to now, none of these two groups are able to crystallize the Az-ODC or Az-AzI complex. In fact, Kahana's group may have moved away from crystallographic approach and started to adopt indirect methods such as alanine-scanning and in silico docking (Cohavi et al., 2009) Two experimental strategies may have given us an edge in this project. Firstly, we have developed a system that allows co-expression of Az with either ODC or AzI in *E. coli*, therefore large amount of structurally homogeneous soluble heterodimers can be readily produced.

Secondly, based on sequence and structural analyses, we have introduced key truncations/internal deletions that effectively facilitate crystallization without compromising the stability of both heterodimers. Since we have already obtained preliminary structures of the ODC-Az and AzI-Az complexes at 2.6 and 5.8 Å resolution, respectively, we are likely to be the first group to publish these two highly significant structures.

2. We have also initiated structural studies on other Az-interacting proteins, including cyclin D1 and Aurora Akinase. Therefore there is a chance for my lab to become a key player in this research field.

3. A research team led by Prof. Kun-Hui Yeh (Department of Oncology, National Taiwan University Hospital) has been studying the effects of ODC overexpression in various gastric and pancreatic cancers. Any small molecule ODC-targeting compounds developed from our study can be tested for their therapeutic potential via collaboration with Prof. Yeh's group. Therefore, this proposal bears a direct medical relevance.

II-3. Overview on the function and molecular mechanism of Az-mediated protein degradation pathway

ODC (EC4.1.1.17) catalyzes the decarboxylation of ornithine to form putrescine, which represents the first and rate-limiting reaction in polyamine biosynthesis (Fig. 21). Subsequent addition of aminopropyl groups by downstream enzymes converts putrescine to spermidine and then to spermine (Seiler *et al.*, 1998). By carrying multiple primary amine groups, the positively charged polyamines may regulate the functions of proteins and nucleic acids by interacting with acidic surface patches of protein and phosphoribosyl backbones of DNA and RNA (Fig. 22), and are thus required for cell growth and differentiation (Tabor & Tabor, 1984). Cellular ODC activity increases rapidly and transiently in response to proliferative or toxic stimuli (Pegg & McCann, l982). Both genetic and pharmacologic experimental approaches have demonstrated that expression of ODC is essential for cell proliferation.

Fig. 21. Structures and biosynthesis pathway of polyamines. (Adopted from *J. Biol. Chem.* **281**:14529-532 (2006)

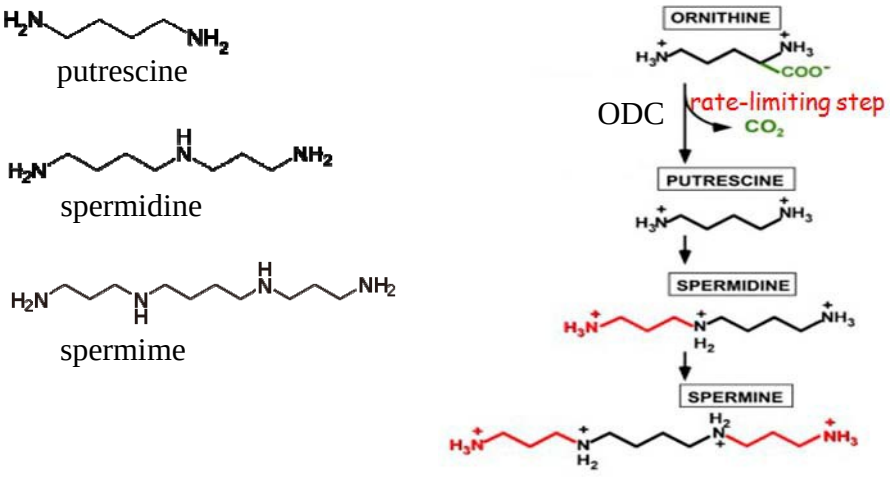
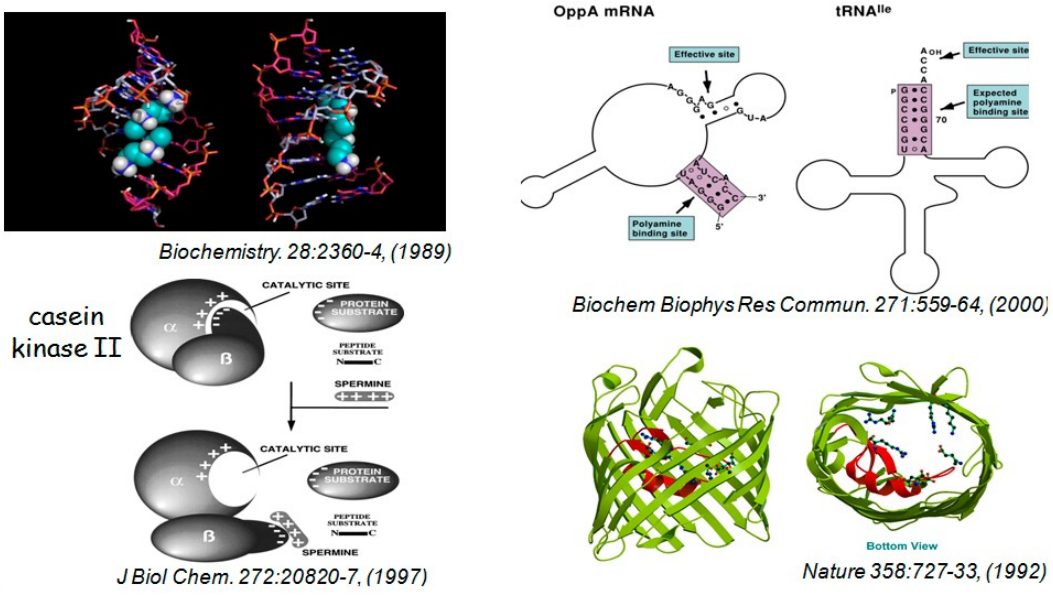


Fig. 22. Activities of proteins and nucleic acids (both DNA and RNA) may be regulated by polyamines.



ODC expression levels are transiently elevated upon stimulation by growth factors. It has been observed that ODC becomes constitutively activated when cells are transformed by carcinogens (Gilmour *et al*., 1987), viruses (Don & Bachrach, 1975; Haddox *et al*., 1980) or oncogenes (Hölttä *et al*., 1988; Chang *et al*., 1988; Sistonen *et al*., 1989). Furthermore, it has been demonstrated that overexpression of ODC led to cellular transformation (Auvinen *et al*., 1992) and tumorigenesis (O'Brien *et al*., 1997). Because ODC is ubiquitous and has been identified as oncogenic, inhibitors of ODC may be therapeutically useful with antitumor activities.

Eukaryotic ODC protomer contains 461 amino acid residues. Enzymatic active ODC exists as a 106 kDa pyridoxal 5'-phosphate (PLP)-dependent homodimer with a molecular 2-fold symmetry (Kitani & Fujisawa, 1984; Solano *et al*., 1985; Mitchell *et al*., 1988). This dimer contains two active sites, each are formed at the interface between the N-terminal domain of one protomer, which provides residues involved in PLP-interactions, and the C-terminal domain of the other protomer with residues interacting with substrate (Kern *et al*., 1999; Osterman *et al*., 1995a, b, 1997; Coleman *et al*., 1993; Tobias & Kahana, 1993; Tsirka & Coffino, 1992).

Some essential amino acid residues in the active site have been identified. In particular, Lys69, Lys169 and His197 from one subunit and Cys360 from the other subunit made up a functional active site (Poulin *et al*., 1992; Tsirka *et al*., 1993; Lu *et al*., 1991; Tsirka, & Coffino, 1992; Coleman *et al*., 1993; Tobias *et al*., 1993). Lys69 is responsible for the formation of a Schiff base with PLP (Poulin *et al*., 1992; Tsirka *et al*., 1992). It plays a key role in catalysis through acceleration of the Schiff base formation, decarboxylation and product release steps (Osterman *et al*., 1999). The role of Cys360 is thought to involve in the reaction chemistry and is essential for efficient product formation (Jackson *et al*., 2000).

ODC activity is modulated by several mechanisms including 1) regulation of ODC transcription (Abrahamsen *et al*., 1992; Abrahamsen & Morris, 1991; Katz & Kahana, 1987), 2) post-translational modification of ODC (Rosenburg-Hasson *et al*., 1991; Worth *et al*., 1994; Kilpeläinen & Hietala, 1994, Reddy *et al*., 1996), and 3) Az-induced but ubiquitin-independent degradation of ODC by the 26S proteasome (Coffino, 1998; Murakami *et al*., 1996; Kanamoto *et al*., 1986; Hayashi *et al*., 1985; Li &

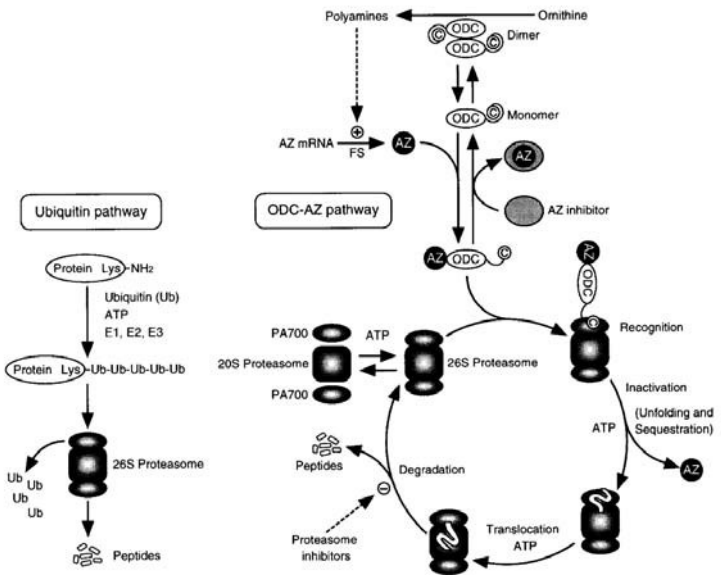
Coffino, 1993). Regulation of ODC enzyme expression at the levels of DNA transcription, mRNA translation, and protein turnover have been described (for reviews, see Refs. Pegg, 1988, & Kameji, *et al.*, 1993). As mentioned above, this proposal focuses exclusively on Az-induced regulatory pathway.

The protein level of Az is tightly and auto-regulated by cellular polyamine concentration. According to a well established model, elevated polyamine concentration causes +1 frameshift of Az mRNA, which allows ribosome to bypass a premature stop colon and produce full-length Az (Matsufuji *et al*., 1995). In addition, it has been suggested very recently that the nascent antizyme polypeptide may serve as an on-site polyamine sensor to suppress the translation of Az mRNA as it is being translated by the ribosome (Kurian *et al*., 2011). Upon its production, AZ selectively binds to the inactive ODC monomer (Li & Coffino, 1992; Mitchell & Chen, 1990), and the formation of the ODC:AZ heterodimer targets ODC for degradation by the 26S proteasome (Coffino, 1998; Murakami *et al*., 1996) (Fig. 23).

Finer mapping of Az fragments indicated that the C-terminal half of Az alone can inactivate ODC and alter its conformation, but it cannot direct degradation of the enzyme, either *in vitro* or *in vivo*. A portion of the N-terminal half of Az must be present to promote degradation (Li & Coffino, 1994). Likewise, the binding of Az to the N-terminus of ODC is essential but not sufficient for degradation (Li & Coffino, 1992), and a second structural element present at the C-terminus is required for the degradation process. Current model states that this interaction between Az and N-terminus of ODC induces a conformational change in ODC, which exposes its C-terminus degradation signal, together with the N-terminal region of Az, the Az-ODC complex binds 26S proteasome with high affinity (Li & Coffino, 1993).

In addition to Az, three other isoforms (Az2, Az3, Az4) have been identified in human. While all members of Az family possess ODC-binding and inhibition activity, interestingly, only Az (commonly referred as Az1) is capable of inducing proteasomal degradation of ODC. It has been suggested that the other Az isoforms may sequester ODC from being degraded by the proteasome, which allows a more rapid restoration of ODC activity when needed. Given the high degree of sequence similarity among Az isoforms, a legitimate structural question is why the heterodimers formed between ODC and the other Az isoforms are spared by the proteasome. Answers to this question should strengthen our understanding on the recognition codes of the 26S proteasome.

Fig. 23. Reguation of ODC activity.
(adopted from Murakami *et al*., 1996)



In addition to the Az-mediated negative regulatory mechanism, the cellular ODC activity is positively regulated by the expression of antizyme inhibitor (AzI), a 50 kDa cytosolic protein. AzI is homologous to ODC but does not possess any decarboxylase activity. AzI inactivates all members of the Az family (Mangold & Leberer 2005), reactivates ODC and prevents the proteolytic degradation of ODC, which may suggest a role for AzI in tumor progression. It has been reported that down regulation of AzI is associated with inhibition of cell proliferation and reduced ODC activity, presumably through modulation of Az function (Choi *et al*., 2005). Moreover, it was shown that overexpression of AzI increases cell proliferation and promotes cell transformation (Keren-Paz *et al*., 2006; Kim *et al*., 2006). Furthermore, AzI is capable of direct interaction with cyclin D1 to prevent its degradation, and this effect is at least partially independent of Az (Mangold 2006). These results demonstrate a role for AzI in the positive regulation of cell proliferation and tumorigenesis. that is homologous to ODC but lacks enzymatic activity. AzI restores cellular ODC level by disrupting the Az-ODC complex through its tight association with Az. Interestingly, while AzI shares ~50% similarity with ODC, the formation of Az-AzI complex does not target AzI for degradation. Instead, Az-binding suppresses an ubiquitin-dependent degradation of AzI. Therefore, it would be both interesting and important to know how Az binding may lead to distinct biological consequences.

The crystal structure of ODC from human liver has been determined to 2.1 Å resolution (Almrud *et al.,* 2000). The human ODC model includes several regions including two degradation elements located in the C-terminus necessary for Az-induced proteolysis. Either of basal degradation elements of ODC have been demonstrated to independently collaborate with Az binding to target ODC for degradation by the 26S proteasome (Coffino, 1998; Li & Coffino, 1993; Ghoda *et al*., 1992; Almrud *et al.,* 2000). In addition, a high resolution crystal structure of AzI and a solution structure of an Az fragment (residues 87~227) have also been reported (Albeck *et al*., 2008; Hoffman *et al*., 2005). Based on these structures, positively charged surfaces regions have been predicted as potential Az-binding elements on both ODC and AzI. However, the proposed electrostatic interactions with Az failed to provide explain the highly specific and salt-resistant nature of both heterodimers. Moreover, a significant portion of the Az solution structure is composed of loop without forming repeatitive secondary structures, suggesting the free Az is likely very flexible and mobile in solution, which makes in silico modeling of Az-ODC and Az-AzI dimers extremely unreliable. Experimentally determined structures are therefore essential for understanding the nature of Az-mediated molecular recognition.

II-4. Long term goals and specific aims

The overall goal of this project aims on elucidating the mechanisms by which Az (and Az isoforms) interact with its target proteins, including ODC, AzI, cyclin D1, and Aurora A kinase, and how the resulting protein complexes are (or are not) recognized by 26S proteasome. Outstanding questions to be addressed include: 1) How does Az recognize its target proteins? 2) How does Az-binding trigger subsequent proteasomal degradation of its target proteins? 3) Why does Az exhibit higher affinity for AzI than ODC despite their high similarity? 4) Why is AzI NOT targeted for degradation upon Az binding? 5) How do the other Az family members interact with the canonical Az-targeting proteins? 6) What are the recognition codes that govern the interactions between Az-associated protein complexes and 26S proteasome? Answers to these questions will no doubt allow better understanding on the various Az-regulated pathways, this work might also have a medical relevance by facilitating the design of small molecule compounds that may bind and trigger similar Az-induced conformational changes in ODC to promote its destruction? Toward this

goal, the following specific aims were proposed:

1. Determine the crystal structure of Az-ODC

As mentioned earlier, we have obtained diffracting crystals of ODC in complex with the C-terminal domain of Az (Az_{95-228}), and the structure has been determined at 2.6 Å resolution. This result not only provides significant insights into the mechanism by which ODC is recognized and targeted for degradation upon Az-binding, more importantly, it lays a solid technical ground for all our subsequent analysis. The established protocols for reconstitution and preparation of ODC- Az_{95-228} can be applied to the other Az target proteins.

2. Determine the crystal structure of Az-AzI

Since preliminary conditions for growing Az-AzI crystals have already been identified, and a preliminary structure has already been determined at 5.8 Å resolution, we will optimize these crystallization conditions by using standard two-dimensional grid refinement to produce single crystals of higher diffraction quality. This structure should reveal why AzI exhibits higher affinity toward Az, and why the resulting complex cannot be recognized by the 26S proteasome.

3. Determine the crystal structures of Az in complexes with cyclin D1 and Aurora A kinase.

We have constructed expression plasmids for both of these Az targets, and the preparation of cyclin D1-Az and Aurora A kinase-Az heterodimer is currently underway. Upon completion, these two structures should offer a more complete view of how Az interacts with its supposedly structurally distinct target proteins.

4. Determine the crystal structures of the various Az target proteins in complex with Az isoforms.

We have already constructed the expression plasmid for Az2, and the cloning of Az3 and Az4 are in progress. As the resulting complexes are not targeted for proteasomal degradation, thus by comparing with the Az-involved complexes, this aim is designed to reveal the structural determinants for proteasome targeting.

5. Determine the crystal structure of 19S regulatory particle or 26S proteasome trapped by the non-degrading ODC- Az_{95-228} heterodimer.

Structural analysis of the 19S regulatory particle and 26S proteasome (19S + 20S) has been discouraged by the fact that certain components of the 19S are loosely or transiently attached, making the preparation of a homogeneous sample very difficult. It has been shown that the presence of substrate protein may trap the proteasome in certain functional states. Unfortunately, the polyubiquitin chains assembled on substrate proteins are heterogeneous in terms of both length and structure. Therefore, the preparation of a large amount of homogeneous sample, with the simultaneous presence of a substrate protein and either the 19S or 26S particle, for crystallization is extremely difficult. In this regard, the use of ODC-Az95-28, which can stably associate with the proteasome without being degraded, may lock the proteasome in a pre-degradation state and thus facilitate structural analysis of either 19S regulatory particle or even the 26S proteasome. This structure would certainly fill a much sought void in the proteasome field.

6. Verify the identified Az-binding elements by mutagenesis studies

After the structures of Az-ODC and Az-AzI are determined, we will use site directed mutagenesis to

examine the effects of interacting residues on the stability of these heterodimers. Gel-filtration and analytical ultracentrifugation will also be conducted when applicable. In addition, by collaborating with Prof. Hui-Chi Hung (National Chung Hsing University), the effects of these mutations on the Az-mediated inhibition of ODC activity and in vitro ODC degradation assay will also be performed.