Critical Factors Determining Dimerization of Human Antizyme Inhibitor

Received for publication, April 14, 2009, and in revised form, June 9, 2009 Published, JBC Papers in Press, July 27, 2009, DOI 10.1074/jbc.M109007807

Kuo-Liang Su†, Ya-Fan Liao§, Hui-Chih Hung††, and Guang-Yaw Liu‡‡

From the †Department of Life Sciences and Institute of Genomics and Bioinformatics, National Chung-Hsing University, Taichung 40227 and the ‡Institute of Immunology, Chung-Shan Medical University, and the ††Division of Allergy, Immunology, and Rheumatology, Chung-Shan Medical University Hospital, Taichung 40201, Taiwan

Ornithine decarboxylase (ODC) is the first enzyme involved in polyamine biosynthesis, and it catalyzes the decarboxylation of ornithine to putrescine. ODC is a dimeric enzyme, whereas antizyme inhibitor (AZI), a positive regulator of ODC that is homologous to ODC, exists predominantly as a monomer and lacks decarboxylase activity. The goal of this paper was to identify the essential amino acid residues that determine the dimerization of AZI. The nonconserved amino acid residues in the putative dimer interface of AZI (Ser-277, Ser-331, Glu-332, and Asp-389) were substituted with the corresponding residues in the putative dimer interface of ODC (Arg-277, Tyr-331, Asp-332, and Tyr-389, respectively). Analytical ultracentrifugation analysis was used to determine the size distribution of these AZI mutants. The size-distribution analysis data suggest that residue 331 may play a major role in the dimerization of AZI. Mutating Ser-331 to Tyr in AZI (AZI-S331Y) caused a shift from a monomer configuration to a dimer. Furthermore, in comparison with the single mutant AZI-S331Y, the AZI-S331Y/D389Y double mutant displayed a further reduction in the monomer-dimer Kd, suggesting that residue 331 is also crucial for AZI dimerization. Analysis of the triple mutant AZI-S331Y/D389Y/S277R showed that it formed a stable dimer (Kd value = 1.3 μM). Finally, a quadruple mutant, S331Y/D389Y/S277R/E332D, behaved as a dimer with a Kd value of ~0.1 μM, which is very close to that of the human ODC enzyme. The quadruple mutant, although forming a dimer, could still be disrupted by antizyme (AZ), further forming a heterodimer, and it could rescue the AZ-inhibited ODC activity, suggesting that the AZ-binding ability of the AZI dimer was retained.

Polyamines (putrescine, spermidine, and spermine) have been shown to have both structural and regulatory roles in protein and nucleic acid biosynthesis and function (1–3). Ornithine decarboxylase (ODC, EC 4.1.1.17) is a central regulator of cellular polyamine synthesis (reviewed in Refs. 1, 4, 5). This enzyme catalyzes the pyridoxal 5-phosphate (PLP)-dependent decarboxylation of ornithine to putrescine, and it is the first and rate-limiting enzyme in polyamine biosynthesis (2, 3, 6, 7). ODC and polyamines play important roles in a number of biological functions, including embryonic development, cell cycle, proliferation, differentiation, and apoptosis (8–15). They also have been associated with human diseases and a variety of cancers (16–26). Because the regulation of ODC and polyamine content is critical to cell proliferation (11), as well as in the origin and progression of neoplastic diseases (23, 24), ODC has been identified as an oncogenic enzyme, and the inhibitors of ODC and the polyamine pathway are important targets for therapeutic intervention in many cancers (6, 11).

ODC is ubiquitously found in organisms ranging from bacteria to humans. It contains 461 amino acid residues in each monomer and is a 106-kDa homodimer with molecular 2-fold symmetry (27, 28). Importantly, ODC activity requires the formation of a dimer (29–31). X-ray structures of the ODC enzyme reveal that this dimer contains two active sites, both of which are formed at the interface between the N-terminal domain of one monomer, which provides residues involved in PLP interactions, and the C-terminal domain of the other subunit, which provides the residues that interact with substrate (27, 32–41).

ODC undergoes a unique ubiquitin-independent proteasomal degradation via a direct interaction with the regulatory protein antizyme (AZ). Binding of AZ promotes the dissociation of the ODC homodimers and targets ODC for degradation by the 26 S proteasome (42–46). Current models of antizyme function indicate that increased polyamine levels promote the fidelity of the AZ mRNA translational frameshift, leading to increased concentrations of AZ (47). The AZ monomer selectively binds to dimeric ODC, thereby inactivating ODC by forming inactive AZ–ODC heterodimers (44, 48–50). AZ acts as a regulator of polyamine metabolism that inhibits ODC activity and polyamine transport, thus restricting polyamine levels (4, 5, 51, 52). When antizymes are overexpressed, they inhibit ODC and promote ubiquitin-independent proteolytic degradation of ODC. Because elevated ODC activity is associ...
AZI, which inactivates all members of the AZ family (53, 56), restores ODC activity (54), and prevents the proteolytic degradation of ODC, may play a role in tumor progression. It has been reported that down-regulation of AZI is associated with the inhibition of cell proliferation and reduced ODC activity, presumably through the modulation of AZ function (57). Moreover, overexpression of AZI has been shown to increase cell proliferation and promote cell transformation (58–60). Furthermore, AZI is capable of direct interaction with cyclin D1, preventing its degradation, and this effect is at least partially independent of AZ function (60, 61). These results demonstrate a role for AZI in the positive regulation of cell proliferation and tumorigenesis.

As mentioned in the previous paragraph, AZI is homologous to the enzyme ODC. It is a 448-amino acid protein with a molecular mass of 50 kDa. However, AZI, which inactivates all members of the AZ family (53, 56), restores ODC activity (54), and prevents the proteolytic degradation of ODC, may play a role in tumor progression. It has been reported that down-regulation of AZI is associated with the inhibition of cell proliferation and reduced ODC activity, presumably through the modulation of AZ function (57). Moreover, overexpression of AZI has been shown to increase cell proliferation and promote cell transformation (58–60). Furthermore, AZI is capable of direct interaction with cyclin D1, preventing its degradation, and this effect is at least partially independent of AZ function (60, 61). These results demonstrate a role for AZI in the positive regulation of cell proliferation and tumorigenesis.

It is now known that ODC exists as a dimer and that AZI may exist as a monomer physiologically (62). In this study, we identify the critical amino acid residues governing the difference in dimer formation between ODC and AZI. Our preliminary studies using analytical ultracentrifugation indicated that ODC exists as a dimer, whereas AZI exists in a concentration-dependent monomer-dimer equilibrium. Multiple sequence alignments of ODC and AZI from various species have shown that residues 277, 331, 332, and 389 are not conserved between ODC and AZI (Table 1). In this study, site-directed mutagenesis was used to generate a series of interface mutants of AZI. Size-distribution analysis of these AZI mutants clearly indicates that a stable AZI dimer similar to the ODC dimer has been successfully obtained.

### MATERIALS AND METHODS

**Expression and Purification of Recombinant Human ODC AZ and AZI**—The human ODC, AZ, and AZI cDNAs were cloned into the pQE30 vector (Qiagen, Hilden, Germany), which carries an N-terminal His-Tag sequence that is useful in the purification of these overexpressed proteins. Expression vectors were transformed into the JM109 strain of Escherichia coli. Protein expression was induced with 1.0 mM isopropyl 1-thiogalactoside, and the cells were grown at 25 °C overnight. Before purification, the cell pellets were dissolved in 25 ml of binding buffer (5 mM imidazole, 500 mM sodium chloride, 30 mM Tris-HCl, pH 7.6) containing 2 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and 0.2% Triton X-100. Nickel-nitrotriacetic acid-Sepharose (3 ml) was then used to purify the His-Tag proteins. HIS-Select Nickel Affinity Gel (Sigma) was first equilibrated with the binding buffer and was then mixed with the cleared lysate at 4 °C for 60 min. The lysate-nickel/gel mixture was loaded into a column and was washed with 100 ml of binding buffer and 100 ml of wash buffer (10 mM imidazole, 500 mM sodium chloride, and 30 mM Tris-HCl, pH 7.6). Finally, all proteins were eluted in elution buffer (250 mM imidazole, 500 mM sodium chloride, and 30 mM Tris-HCl, pH 7.6) containing 0.2 M imidazole.

---

**TABLE 1**

| Amino acid residues at the dimer interface of human ODC and AZI |
|---------------------------------------------------------------|
| **Nonconserved**                                              |
| Arg               277                    Ser               |
| Tyr               331                    Ser               |
| Asp               332                    Glu               |
| Tyr               389                    Asp               |
| **Conserved**                                                |
| Asp               134                    Asp               |
| Lys               169                    Lys               |
| Lys               294                    Lys               |
| Tyr               323                    Tyr               |
| Asp               364                    Asp               |
| Gly               387                    Gly               |
| Phe               397                    Phe               |

---
500 mM sodium chloride, 30 mM Tris–HCl, pH 7.6) with 2 mM β-mercaptoethanol. After a stepwise wash procedure, the proteins were eluted and concentrated for further experiments. The purity of all recombinant proteins was estimated via SDS-PAGE to be about 95%.

**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out using the QuikChange kit (Stratagene, La Jolla, CA). This mutagenesis method uses *Pfu* DNA polymerase, which replicates both plasmid strands with high fidelity in a 16–20-cycle PCR. Primers with 28–34 bases in length that include the mutations were used for the specific amplification of template DNA. The PCR primers used were as follows: 5′-GATAATTTCAGAACCCGGAACGTACTATGTGTCCTTCTGC-3′ for AZI-S277R; 5′-GTTCTTTTGCAAGTAACTGTACGAGGACTTAAATCCATC-3′ for AZI-S331Y; 5′-GCTATGACTGAGCTTAATACCATTCC-3′ for AZI-S331Y; 5′-GCAAAGTAAACCTGTGACGACTTAATACCATTCC-3′ for AZI-E332D; and 5′-CTTTGATAACATGGGAGCATCTCCTTCCATGAACCATTGC-3′ for AZI-D389Y. The mutation positions in the oligonucleotide sequence are underlined and in boldface. The PCR conditions were 95 °C for 30 s, 55 °C for 1 min, and 68 °C for a period equivalent to 2 min/kb of plasmid length, for a total of 16 cycles. The PCR products were subsequently treated with DpnI to digest the wild-type human AZI templates. Finally, the nicked DNA with the desired mutations was transformed into the XL-1 *E. coli* strain (Stratagene, La Jolla, CA), and the DNA sequences were checked by autosequencing.

**Enzyme Assay and Kinetic Analysis**—The ODC enzyme activity was measured at 37 °C using the CO₂-L3K assay kit (DCL, Charlottetown, Canada). The enzyme assay couples the decarboxylation of ornithine to the carboxylation of phosphoenolpyruvate to form oxaloacetate, which becomes malate following NADH oxidation. The standard reaction mixture for a spectrophotometric assay of ODC contains 30 mM Tris–HCl, pH 7.8, 10 mM ornithine, 0.2 mM PLP, and 0.4 ml of CO₂-L3K assay buffer containing 12.5 mM phosphoenolpyruvate, 0.4 unit/ml microbial phosphoenolpyruvate carboxylase, 4.1 units/ml malate dehydrogenase (mammalian malate dehydrogenase),

![FIGURE 2. Continuous sedimentation coefficient distribution of the human ODC-WT, AZI-WT, and AZI single mutants. The proteins used are at three protein concentrations, 0.3, 0.6, and 0.9 mg/ml in 50 mM Tris–HCl buffer, pH 7.4, at 20 °C. A, ODC-WT; B, AZI-WT; C, AZI-S331Y; D, AZI-D389Y; E, AZI-S277R; F, AZI-E332D. M, monomer; D, dimer.](image-url)
and 0.6 mM NADH analog in a final volume of 0.5 ml. An appropriate amount of ODC was then added to the assay mixture to initiate the reaction. After the addition of enzyme to the reaction mixture, the decrease of absorbance at 405 nm was continuously traced in a Lambda 25 UV-visible spectrophotometer (PerkinElmer Life Sciences). In this coupled assay method, 1 mol of CO₂ was produced, and 1 mol of NADH analog was oxidized under the assay conditions. An enzyme unit is defined as the amount of enzyme that catalyzes the production of 1 μmol of NAD per min. An absorption coefficient of 2410 M⁻¹ cm⁻¹ was used for the NADH analog in the calculations. All of the calculations were performed using the SigmaPlot 10.0 software program (Jandel, San Rafael, CA).

**Text Table**

| Protein          | \( K_d \) | Protein          | \( K_d \) |
|------------------|-----------|------------------|-----------|
| ODC-wt           | 0.18 ± 0.0009 | AZI-S331Y/E332D | 31.0 ± 0.23 |
| AZI-wt           | 84.0 ± 0.44  | AZI-S277R/D389Y | 431.1 ± 0.38 |
| AZI-S331Y        | 40.7 ± 0.20  | AZI-D389Y/E332D | 107.2 ± 0.92 |
| AZI-D389Y        | 71.9 ± 0.55  | AZI-S277R/E332D | 110.9 ± 0.95 |
| AZI-S277R        | 105.2 ± 0.77 | AZI-S331Y/D389Y/S277R | 1.3 ± 0.01 |
| AZI-E332D        | 134.9 ± 1.13 | AZI-S331Y/D389Y/E332D | 5.9 ± 0.07 |
| AZI-D389Y/S277R  | 2.7 ± 0.03   | AZI-S331Y/S277R/E332D | 20.3 ± 0.22 |
| AZI-S331Y/S277R  | 10.8 ± 0.05  | AZI-S331Y/D389Y/S277R/E332D | 0.10 ± 0.0006 |

*The \( K_d \) value was derived from global data fitting of the sedimentation velocity at three different protein concentrations for each protein.*

![Continuous sedimentation coefficient distribution for the six double mutants of human AZI](image)

**FIGURE 3.** Continuous sedimentation coefficient distribution for the six double mutants of human AZI. The proteins used are at three protein concentrations, 0.3, 0.6, and 0.9 mg/ml in 50 mM Tris-HCl buffer, pH 7.4, at 20 °C. A, AZI-S331Y/S277R; B, AZI-S331Y/E332D; C, AZI-S331Y/D389Y; D, AZI-D389Y/S277R; E, AZI-S277R/E332D; F, AZI-D389Y/E332D. M, monomer; D, dimer.
drous frictional ratio \( (f/f_0) \), and a resolution \( N \) of 250 sedimentation coefficients between 0.1 and 20.0 S.

To determine precisely the dissociation constants of ODC and AZI mutants in monomer-dimer equilibrium, sedimentation velocity experiments were performed at three different protein concentrations for each protein. To calculate dissociation constants \( (K_d) \), all sedimentation data were globally fit into the monomer-dimer equilibrium model using the program SEDPHAT (65). Furthermore, to determine the dissociation constants of the human ODC and AZI proteins toward AZ, sedimentation velocity experiments were performed at five different concentrations of AZ in the presence of a constant concentration of human ODC or AZI. To calculate the \( K_d \) value of these heterodimers, all sedimentation data were globally fit into the AB hetero-association model using the program SEDPHAT (65). The partial specific volumes of the proteins, the solvent densities, and the viscosity were calculated by the software program SEDNTERP (67).

**RESULTS**

**Size-distribution Analysis of the Wild-type and Dimer Interface Mutants of Human AZI**—To identify amino acid residues that are required for the dimerization of AZI, a series of dimer interface mutants of AZI was created. Nonconserved amino acid residues in the putative dimer interface of AZI (Ser-277, Ser-331, Glu-332, and Asp-389) were substituted with the corresponding amino acid residues at the respective positions in ODC (Arg-277, Tyr-331, Asp-332, and Tyr-389, respectively). Analytical ultracentrifugation analysis was used to examine the size distribution of the AZI mutants. By global fitting of the sedimentation velocity data, the dissociation constants of the wild-type and interface mutants of human AZI were obtained.

Size-distribution analysis of ODC-WT and AZI-WT indicated that ODC is a dimer (Fig. 2A) with a \( K_d \) value of 0.18 \( \mu \)M in monomer-dimer equilibrium (Table 2), whereas AZI is in rapid equilibrium between monomer and dimer configurations (Fig. 2B) with a dissociation constant of 84 \( \mu \)M (Table 2). The dissociation constant of AZI, which is larger than that of ODC by 460-fold, indicates a weak interaction at the dimer interface of AZI. Mutation of Ser-331 to Tyr in AZI (AZI-S331Y) resulted in a shift from monomer to dimer (Fig. 2C) with a \( K_d \) value of 41 \( \mu \)M (Table 2), a 2-fold decrease compared with AZI-WT protein. The AZI-D389Y protein displayed a small shift in monomer-dimer equilibrium (Fig. 2D), with a \( K_d \) value of 72 \( \mu \)M (Table 2). The AZI-S277R and AZI-E332D mutants, however, did not show a shift from monomer to dimer (Fig. 2, E and F, respectively), and it displayed larger \( K_d \) values than that of AZI-WT. These data suggest that residue 331 may play a significant role in AZI dimerization.

The size distribution of the six double mutants of AZI further revealed the importance of residue 331 in the dimerization of AZI. The S331Y-containing AZI double mutants (S331Y/S277R, S331Y/E332D, and S331Y/D389Y) resulted in a shift from monomer to dimer (Fig. 2C) with a \( K_d \) value of 41 \( \mu \)M (Table 2), a 2-fold decrease compared with AZI-WT protein. The AZI-D389Y protein displayed a small shift in monomer-dimer equilibrium (Fig. 2D), with a \( K_d \) value of 72 \( \mu \)M (Table 2). The AZI-S277R and AZI-E332D mutants, however, did not show a shift from monomer to dimer (Fig. 2, E and F, respectively), and it displayed larger \( K_d \) values than that of AZI-WT. These data suggest that residue 331 may play a significant role in AZI dimerization.

The size distribution of the six double mutants of AZI further revealed the importance of residue 331 in the dimerization of AZI. The S331Y-containing AZI double mutants (S331Y/S277R, S331Y/E332D, and S331Y/D389Y) demonstrated significant shifts in equilibrium from monomers to dimers (Fig. 3, A–C, respectively), with significant decreases in \( K_d \) values (11, 31, and 2.7 \( \mu \)M, respectively, see Table 2). For AZI-S331Y/D389Y in particular, little monomer existed at equilibrium; most AZI monomers became dimers (Fig. 3C), similar to the size-distribution pattern of ODC-WT (Fig. 2A). Furthermore,
the $K_d$ value of the S331Y/D389Y double mutant (2.7 $\mu M$) displayed a significant reduction in comparison with the single mutant AZI-S331Y (41 $\mu M$) and was about 31-fold lower than the AZI-WT (84 $\mu M$), suggesting that residue 389 plays a crucial secondary role in the dimerization of AZI. The AZI-D389Y/S277R double mutant, although lacking S331Y, still showed a significant shift in the monomer-dimer equilibrium (Fig. 3D) with a $K_d$ value of 43 $\mu M$ (Table 2). The fact that the $K_d$ value for this double mutant is similar to that of AZI-S331Y suggests a supplementary role for residues 389 and 277. The double mutants AZI-S277R/E332D and AZI-D389Y/E332D showed no shift in monomer-dimer equilibrium (Fig. 3, E and F, respectively); the $K_d$ values of these two mutants were ~110 $\mu M$ (Table 2), even larger than that of AZI-WT.

Further analysis of the size distribution for the triple mutant AZI-S331Y/D389Y/S277R supports an essential role for these residues in formation of an AZI dimer. Size-distribution analysis of this triple mutant indicated that it formed a stable dimer (Fig. 4A) with a $K_d$ value of 1.3 $\mu M$ (Table 2), a further 2-, 8-, and 33-fold decrease compared with the AZI double mutants S331Y/D389Y (2.7 $\mu M$), S331Y/S277R (11 $\mu M$), and D389Y/S277R (43 $\mu M$), respectively. The other triple mutants containing the E332D mutation, AZI-S331Y/D389Y/E332D and AZI-S331Y/S277R/E332D (Fig. 4, B and C, respectively), showed $K_d$ values of 6 and 20 $\mu M$, respectively, larger than those of the respective double mutants lacking E332D (S331Y/D389Y and S331Y/S277R). This indicates that residue 332 is not a major factor in determining the dimeric structure formation of AZI.

Although AZI-S331Y/D389Y/S277R exists in a stable dimeric form, its $K_d$ value is still higher than that of ODC by nearly 1 order of magnitude. We further analyzed the dissociation constant of the quadruple mutant AZI-S331Y/D389Y/S277R/E332D (Fig. 4D), finally obtaining an AZI dimer protein with a $K_d$ value about 0.1 $\mu M$ (Table 2), which is very close to that of the human ODC enzyme.

**Functional Characterization of the AZI-S331Y/D389Y/S277R/E332D Dimer**—The AZI-S331Y/D389Y/S277R/E332D protein displayed a dimeric structure similar to the ODC enzyme. To investigate the AZ-binding ability of this AZI dimer, we examined the quaternary structure of human ODC-WT, AZI-WT, and AZI-S331Y/D389Y/S277R/E332D proteins in the presence of AZ (Fig. 5). In addition, the dissociation constants of the human ODC and AZI proteins toward AZ were also determined (Table 3).

The molar ratio of the ODC or AZI monomer versus AZ monomer in this experiment was about 1:1. The size distribution of the dimeric ODC without (Fig. 5A, solid line) or with AZ (Fig. 5A, dashed line) clearly showed that ODC-WT was dissociated in the presence of AZ and that it bound with AZ to form ODC-AZ heterodimers. The peak of the ODC-AZ heterodimer lies to the left of that of the ODC dimer, and the molecular mass of the ODC-AZ heterodimer is about 80 kDa (Fig. 5A, dashed line), which is smaller than that of dimeric ODC (106 kDa), confirming that the heterodimer consists of an AZ monomer in association with an AZ dimer. The $K_d$ value of the ODC-AZ heterodimer was $0.29 \pm 0.003$ $\mu M$ (Table 3). In contrast to ODC, which exists predominantly as a dimer, the human AZI protein is in rapid monomer-dimer equilibrium and exists predominantly as a monomer at low protein concentration (Fig. 5B, solid line). In the presence of AZ, the AZI monomer bound with AZ to form an AZI-AZ heterodimer (Fig. 5B, dashed line). The peak of the AZI-AZ heterodimer lies to the right of that of the AZI monomer, and the molecular mass of the AZI-AZ heterodimer is about 78 kDa. The $K_d$ value of this AZI-AZ heterodimer was $0.027 \pm 0.0002$ $\mu M$ (Table 3).

![FIGURE 5. Continuous sedimentation coefficient distribution for the human ODC-WT, AZI-WT, and quadruple mutant AZI-S331Y/D389Y/S277R/E332D in the presence of AZ.](image)

**TABLE 3**

| Protein complex | $K_d$ | $\mu M$ |
|-----------------|-------|---------|
| ODC-AZ          | 0.29  | ± 0.003 |
| AZI-AZ          | 0.027 | ± 0.0002 |
| [AZI-S331Y/D389Y/S277R/E332D]-AZ | 0.023 | ± 0.0003 |

* The $K_d$ value was derived from global data fitting of the sedimentation velocity at five different protein concentrations of AZ (0.05–0.3 mg/ml, see supplemental Fig. 1). The protein concentrations of human ODC and AZI were fixed at 0.3 mg/ml.
and furthermore, it can form a heterodimer just like ODC (Fig. 5C, dashed line). The $K_d$ value of the AZI-S331Y/D389Y/S277R/E332D-AZ heterodimer was $0.023 \pm 0.0003 \mu M$ (Table 3), which was very close to that of the AZI-AZ heterodimer, indicating a similar AZ-binding affinity for the AZI-AZ and AZI-AZI-AZ heterodimer. These results suggest that the self-association of AZI did not influence the stability of AZI-AZ heterodimers and imply that the dimerization of AZI does not affect its binding to AZ.

Because AZ causes the dissociation of ODC and only the dimeric form of ODC is enzymatically active, ODC enzyme activity can be inhibited by AZ. Fig. 6A shows that the activity of ODC is gradually lost with increased AZ concentration. Nevertheless, AZI can rescue AZ-inhibited ODC enzyme activity through competitive binding with AZ with respect to ODC. When ODC monomers are released from ODC-AZ heterodimeric complexes by AZI, they rapidly reconstitute into dimers, and the enzyme activity of ODC is recovered. Fig. 6B shows that the AZ-inhibited ODC enzyme activity was recovered in the presence of AZI-WT (open circles).

With increasing AZI-WT concentrations, the residual enzyme activity was elevated from 30 to over 80%. The AZI-inhibited ODC activity can also be recovered by the AZI-S331Y/D389Y/S277R/E332D protein (Fig. 6B, closed circles). The residual enzyme activity curves of the AZI-WT and AZI-S331Y/D389Y/S277R/E332D dimer nearly overlap, indicating that, similar to AZI-WT, the AZI-S331Y/D389Y/S277R/E332D protein still retains the ability to rescue AZ-inhibited ODC enzyme activity.

The ODC enzyme cannot form a heterodimer with the AZI monomer. The addition of AZI in the assay mixture does not inhibit the ODC enzyme (Fig. 7A, open circles). Size-distribution analysis revealed a protein peak for monomeric AZI (left) and dimeric ODC (Fig. 7B). Because the monomeric ODC and dimeric AZI-S331Y/D389Y/S277R/E332D are enzymatically inactive, if the ODC dimer and the AZI dimer exchange their subunits to form a heterodimer, the chimeric ODC-AZI dimer should either be inactive or have its activity reduced by half. However, incubation of ODC with the AZI-S331Y/D389Y/S277R/E332D dimer did not suppress ODC enzyme activity (Fig. 7A, closed circles), suggesting that the ODC and AZI dimers cannot be exchanged to form heterodimers. Size-distribution analysis does not clearly discriminate between the ODC dimer, AZI dimer, and ODC-AZI dimer (if formed) because they are all of very similar sizes.
DISCUSSION

ODC is a key enzyme in the biosynthesis of cellular polyamines, and AZI is a positive regulator of ODC activity. AZI is homologous to ODC; however, ODC is a dimeric enzyme (27, 28), whereas AZI exists predominantly as a monomer and lacks decarboxylase activity (62). The objective of this study was to define the essential amino acid residues that determine why AZI exists as a monomer. In this study, we also demonstrate that an AZI dimer with quadruple mutations still functions as a monomeric AZI protein with respect to AZ-binding ability and positive regulation of ODC enzyme activity.

Factors That Determine the Dimerization of AZI—Based on multiple sequence alignments of ODC and AZI, the nonconserved amino acid residues in the putative dimer interface of AZI (Ser-277, Ser-331, Glu-332, and Asp-389) were substituted with the corresponding amino acid residues of ODC (Arg-277, Tyr-331, Asp-332, and Tyr-389, respectively). These amino acid replacements allowed the AZI protein to form dimers. Based on size-distribution analysis of these AZI interface mutants, we suggest that residue 331 is the most important residue governing the dimerization of human AZI protein. All of the human mutant AZI proteins containing S331Y (S331Y, S331Y/S277R, S331Y/E332D, S331Y/D389Y, S331Y/D389Y/S277R, S331Y/S277R/E332D, S331Y/D389Y/E332D, and S331Y/D389Y/S277R/E332D) have $K_d$ values that are significantly smaller than that of AZI-WT (Table 2). In the structure of human ODC, Tyr-331 from one subunit is hydrogen-bonded with Val-322 of another subunit and may have hydrophobic interactions with amino acid residues from another subunit at the dimer interface (Fig. 8). In human AZI, Tyr-331 is substituted by Ser, which is not involved in these hydrogen-bonding networks and hydrophobic interactions. However, Tyr-331 interactions may be crucial for dimer formation in the AZI mutant protein, whereas the subunit-subunit interface may be closer in the human S331Y mutant of AZI.

Besides residue 331, residue 389 is also a determinant for the dimerization of AZI. In human ODC, this residue is a Tyr; in AZI, it is replaced by Asp. Substitution of Asp-389 by Tyr in

FIGURE 8. LIGPLOT of the amino acid residues at the dimer interface for human ODC. Ligand interactions at the dimer interface residues Tyr-331, Tyr-389, Arg-277, and Asp-332 are shown as the LIGPLOT diagram (68). The boldface bonds indicate the specific amino acid residue; the thin bonds are the hydrogen-bonded residues, and the green dashed lines correspond to the hydrogen bonds. Spoked arcs represent hydrophobic contacts.
Human Antizyme Inhibitor

human AZI did not cause a pronounced shift in equilibrium from monomers to dimers. However, mutation of Asp-389 to Tyr in human AZI-S331Y decreased the $K_d$ value over 15-fold. The fact that the double mutant AZI-S331Y/D389Y showed a further significant decrease in $K_d$ value and that, like the ODC enzyme, it displayed a major peak corresponding to the dimer (Fig. 3C) suggests that residue 389 plays a supplementary role in the dimerization of the human AZI protein. Based on size-distribution analysis, we also found that, like residue 389, residue 277 may play an important auxiliary role in the dimerization of AZI. Mutating Ser-277 to Arg in human AZI-S331Y resulted in a significant decrease in the $K_d$ value, similar to that of AZI-S331Y/D389Y. However, AZI-S331Y/S277R did not completely shift the equilibrium from monomers to dimers (Fig. 4). The electrostatic balance in the putative dimer interface of AZI is also influential in the dimerization of AZI. In the structure of ODC, Arg-277 is ion-paired with Asp-332 (Fig. 8). This ion pair in the AZI-S331Y/D389Y/S277R/E332D dimer may support the formation of a close dimer interface by keeping the electrostatic balance and correct geometry at the dimer interface.

Functional Properties of the Quadruple Mutant S331Y/D389Y/S277R/E332D Human AZI Protein—The fact that the $K_d$ value of the human AZI-S331Y/D389Y/S277R/E332D protein is very close to that of the human ODC enzyme (Table 2) shows that this quadruple mutant has been transformed into a dimeric protein. Although the quadruple mutant AZI protein has become a stable dimer, its AZ-binding affinity seems to be unchanged (Table 3). The evidence for this is 2-fold. First, the dimeric structure of the mutant can be disrupted by AZ, and after binding to AZ, the mutant and AZ form a heterodimer similar to the wild-type AZI-AZ heterodimer. Second, AZ-inhibited ODC enzyme activity is rescued by the mutant AZI dimer, in a way that is similar to the rescue of ODC activity by AZI-WT monomers.

Because AZI is homologous to ODC and has a similar molecular weight, we suggest that the putative AZ-binding site of AZI may be the same as that of the ODC enzyme, which resides in the region of residues 117–140 (28) (Fig. 1). Structural data of AZI reveal that the putative AZ-binding site of AZI is distant from the dimer interface of the protein (Fig. 1). Therefore, the self-association of AZI does not interfere with the AZ-binding affinity, and thus the AZI-mediated relief of AZ-inhibited ODC activity did not decline in response to the enhanced AZI dimerization (Fig. 6B). This suggests that the subunit-subunit interaction of ODC or AZI and the AZ-binding site are independent of each other.

In this study, we have identified the essential residues that determine the quaternary structure of the human AZI protein, and we have successfully produced a stable dimeric AZI protein. Because a dimeric structure is required for ODC activity, the quadruple mutant AZI dimer described here may be useful in further generation of an enzymatically active AZI. The essential amino acid residues that are important for ODC enzyme catalysis and substrate/cofactor binding have been identified, and some of these residues are not conserved in AZI. After the substitution of these amino acid residues as well as those essential for dimerization, a dimeric AZI with decarboxylase activity might ultimately be generated.

REFERENCES

1. Gerner, E. W., and Meyskens, F. L., Jr. (2004) Nat. Rev. Cancer 4, 781–792
2. Pegg, A. E., and McCann, P. P. (1982) Am. J. Physiol. 243, C212–C221
3. Tabor, C. W., and Tabor, H. (1984) Annu. Rev. Biochem. 53, 749–790
4. Mangold, U. (2005) IUBMB Life 57, 617–666
5. Pegg, A. E. (2006) J. Biol. Chem. 281, 14529–14532
6. Seiler, N., Atanasov, C. L., and Raul, F. (1998) Int. J. Oncol. 13, 993–1006
7. Palanimurugan, R., Scheel, H., Hofmann, K., and Dohmen, R. J. (2004) EMBO J. 23, 4857–4867
8. Auvinen, M., Pasisen, A., Andersson, L. C., and Hölttä, E. (1992) Nature 360, 355–358
9. Smith, M. K., Goral, M. A., Wright, J. H., Matrisian, L. M., Morris, R. J., Klein-Szanto, A. J., and Gilmour, S. K. (1997) Cancer Res. 57, 2104–2108
10. O’Brien, T. G., Megosh, L. C., Gilliard, G., and Soler, A. P. (1997) Cancer Res. 57, 2630–2637
11. Thomas, T., and Thomas, T. J. (2001) Cell. Mol. Life Sci. 58, 244–258
12. Pegg, A. E., and McCann, P. P. (1982) Am. J. Physiol. 243, C212–C221
13. Pegg, A. E., and McCann, P. P. (1982) Am. J. Physiol. 243, C212–C221
14. Huang, C. C., Hsu, P. C., Hung, Y. C., Liu, C. C., Hour, C. T., Kao, M. C., Tsay, G. J., Hung, H. C., and Liu, G. Y. (2005) Apoptosis 10, 389–399
15. Hsu, P. C., Hour, T. C., Liao, Y. F., Hung, Y. C., Liu, C. C., Hour, C. T., Kao, M. C., Tsay, G. J., Hung, H. C., and Liu, G. Y. (2005) Apoptosis 10, 895–907
16. Hayashi, S., Kameji, T., Fujita, K., Murakami, Y., Utsunomiya, K., Matsufuji, S., Takiguchi, M., Mori, T., and Tatabana, M. (1995) Adv. Enzyme Regul. 35, 311–329
17. Medina, M. A., Quesada, A. R., Núñez de Castro, I., and Sánchez-Jiménez, F. (1999) Biochem. Pharmacol. 57, 1341–1344
18. Thomson, S. C., Deng, A., Bao, D., Satriano, J., Blantz, R. C., and Vallon, V. (2001) J. Clin. Invest. 107, 217–224
19. Furumitsu, Y., Yukioka, K., Yukioka, M., Ochi, T., Morishima, Y., Matsuyasu, I., Otani, S., Inaba, M., Nishizawa, Y., and Morii, H. (2000) J. Rheumatol. 27, 1352–1357
20. Hsu, H. C., Thomas, T., Sigal, L. H., and Thomas, T. J. (1999) Autoimmunity 29, 299–309
21. Deng, A., Munger, K. A., Valdivielso, J. M., Satriano, J., Lortie, M., Blantz, R. C., and Thomson, S. C. (2003) Diabetes 52, 1235–1239
22. Schlüter, K. D., Frischkopf, K., Flesch, M., Rosenkrantz, S., Taimor, G., and Piper, H. M. (2000) Cardiovasc. Res. 45, 410–417
23. Morrison, L. D., Cao, X. C., and Kish, S. J. (1998) J. Neurochem. 71, 288–294
24. Auvinen, M., Laine, A., Pasisen-Soehns, A., Kangas, A., Kangas, L., Saksela, O., Andersson, L. C., and Hölttä, E. (1997) Cancer Res. 57, 3016–3025
25. Packham, G., and Cleveland, J. L. (1994) Mol. Cell. Biol. 14, 5741–5747
26. Park, J. K., Chung, Y. M., Kang, S., Kim, I. U., Kim, Y. T., Kim, H. J., Kim, Y. H., Kim, J. S., and Yoo, Y. D. (2002) Mol. Pharmacol. 62, 1400–1408
27. Kern, A. D., Oliveira, M. A., Coffino, P., and Hackert, M. L. (1999) Structure 7, 567–581
28. Almud, J. J., Oliveira, M. A., Kern, A. D., Grishin, N. V., Phillips, M. A., and Hackert, M. L. (2000) J. Mol. Biol. 295, 7–16
29. Kitani, T., and Fujisawa, H. (1984) J. Biol. Chem. 259, 10036–10040
30. Solano, F., Peñañuelas, J., Solano, M. E., and Lozano, J. A. (1985) FEBS Lett. 190, 324–328
31. Mitchell, J. L., Rynning, M. D., Hong, H. J., and Hicks, H. F. (1988) Arch.
32. Osterman, A. L., Kinch, L. N., Grishin, N. V., and Phillips, M. A. (1995) *J. Biol. Chem.* 270, 11797–11802
33. Osterman, A. L., Lueder, D. V., Quick, M., Myers, D., Canagarajah, B. J., and Phillips, M. A. (1995) *Biochemistry* 34, 13431–13436
34. Osterman, A. L., Brooks, H. B., Rizo, J., and Phillips, M. A. (1997) *Biochemistry* 36, 4558–4567
35. Coleman, C. S., Stanley, B. A., and Pegg, A. E. (1993) *J. Biol. Chem.* 268, 24572–24579
36. Coleman, C. S., Stanley, B. A., Viswanath, R., and Pegg, A. E. (1994) *J. Biol. Chem.* 269, 3155–3158
37. Tsirka, S., and Coffino, P. (1992) *J. Biol. Chem.* 267, 23057–23062
38. Tobias, K. E., and Kahana, C. (1993) *Biochemistry* 32, 5842–5847
39. Jackson, L. K., Baldwin, J., Akella, R., Goldsmith, E. J., and Phillips, M. A. (2004) *Biochemistry* 43, 12990–12999
40. Poulin, R., Lu, L., Ackermann, B., Bey, P., and Pegg, A. E. (1992) *J. Biol. Chem.* 267, 150–158
41. Jackson, L. K., Goldsmith, E. J., and Phillips, M. A. (2003) *J. Biol. Chem.* 278, 22037–22043
42. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) *Nature* 360, 597–599
43. Hayashi, S., Murakami, Y., and Matsufuji, S. (1996) *Trends Biochem. Sci.* 21, 27–30
44. Coffino, P. (1998) in *Ubiquitin and the Biology of the Cell* (Peters, J. M., Harris, J. R., and Finley, D., eds) pp. 411–427, Plenum Publishing Corp., New York
45. Zhang, M., Pickart, C. M., and Coffino, P. (2003) *EMBO J.* 22, 1488–1496
46. Zhang, M., MacDonald, A. L., Hoyt, M. A., and Coffino, P. (2004) *J. Biol. Chem.* 279, 20959–20965
47. Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F., and Hayashi, S. (1995) *Cell* 80, 51–60
48. Li, X., and Coffino, P. (1992) *Mol. Cell. Biol.* 12, 3556–3562
49. Mitchell, J. L., and Chen, H. J. (1990) *Biochim. Biophys. Acta* 1037, 115–121
50. Coffino, P. (2001) *Biochimie* 83, 319–323
51. Coffino, P. (2001) *Nat. Rev. Mol. Cell Biol.* 2, 188–194
52. Sakata, K., Kashiwagi, K., and Igarashi, K. (2000) *Biochem. J.* 347, 297–303
53. Murakami, Y., Ichiba, T., Matsufuji, S., and Hayashi, S. (1996) *J. Biol. Chem.* 271, 3340–3342
54. Nilsson, J., Grahn, B., and Heby, O. (2000) *Biochem. J.* 346, 699–704
55. Bercovich, Z., and Kahana, C. (2004) *J. Biol. Chem.* 279, 54097–54102
56. Mangold, U., and Leberer, E. (2005) *Biochem. J.* 385, 21–28
57. Choi, K. S., Suh, Y. H., Kim, W. H., Lee, T. H., and Jung, M. H. (2005) *Biochem. Biophys. Res. Commun.* 328, 206–212
58. Keren-Paz, A., Bercovich, Z., Porat, Z., Erez, O., Brener, O., and Kahana, C. (2006) *Oncogene* 25, 5163–5172
59. Kim, S. W., Mangold, U., Waghover, C., Mobascher, A., Shantz, L., Bandyard, J., and Zetter, B. R. (2006) *J. Cell Sci.* 119, 2583–2591
60. Mangold, U. (2006) *Cell. Mol. Life Sci.* 63, 2095–2101
61. Newman, R. M., Mobascher, A., Mangold, U., Koike, C., Diah, S., Schmidt, M., Finley, D., and Zetter, B. R. (2004) *J. Biol. Chem.* 279, 41504–41511
62. Albeck, S., Dym, O., Unger, T., Snapir, Z., Bercovich, Z., and Kahana, C. (2008) *Protein Sci.* 17, 793–802
63. Schuck, P. (2000) *Biophys. J.* 78, 1606–1619
64. Lebowitz, J., Lewis, M. S., and Schuck, P. (2002) *Protein Sci.* 11, 2067–2079
65. Schuck, P. (2003) *Anal. Biochem.* 320, 104–124
66. Schuck, P., Perugini, M. A., Gonzales, N. R., Howlett, G. J., and Schubert, D. (2002) *Biophys. J.* 82, 1096–1111
67. Laue, T. M., Shah, B. D., Ridgeway, T. M., and Pelletter, S. L. (1992) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., and Horton, J. C., eds) The Royal Society of Chemistry, Cambridge, UK
68. Wallace, A. C., Laskowski, R. A., and Thornton, J. M. (1995) *Protein Eng.* 8, 127–134