Atrazine chlorohydrolase, TrzN (triazine hydrolyase or atrazine chlorohydrolase 2), initiates bacterial metabolism of the herbicide atrazine by hydrolytic displacement of a chlorine substituent from the 3-triazine ring. The present study describes crystal structures and reactivity of wild-type and active site mutant TrzN enzymes. The homodimer native enzyme structure, solved to 1.40 Å resolution, is a (βα)8 barrel, characteristic of members of the amidohydrolase superfamily. TrzN uniquely positions threonine 325 in place of a conserved aspartate that ligates the metal in most mononuclear amidohydrolases superfamily members. The threonine side chain oxygen atom is 3.3 Å from the zinc atom and 2.6 Å from the oxygen atom of zinc-coordinated water. Mutation of the threonine to a serine resulted in a 12-fold decrease in $k_{\text{cat}}/K_{\text{m}}$, largely due to $k_{\text{cat}}$, whereas the T325D and T325E mutants had immeasurable activity. The structure and kinetics of TrzN are reminiscent of carbonic anhydrase, which uses a threonine to assist in positioning water for reaction with carbon dioxide. An isosteric substitution in the active site glutamate, E241Q, showed a large diminution in activity with ametryn, no detectable activity with atrazine, and a 10-fold decrease with atrazine, when compared with wild-type TrzN. Activity with the E241Q mutant was nearly constant from pH 6.0 to 10.0, consistent with the loss of a proton-donating group. Structures for TrzN-E241Q were solved with bound ametryn and atratone to 1.93 and 1.64 Å resolution, respectively. Both structure and kinetic determinations suggest that the Glu241 side chain provides a proton to N-1 of the s-triazine substrate to facilitate nucleophilic displacement at the adjacent C-2.

There is substantial evidence that microbes rapidly evolve new enzymes to metabolize anthropogenic chemicals (1, 2). The s-triazine herbicides, such as atrazine, were first introduced into the environment 50 years ago and >2 billion pounds have been applied globally. s-Triazine compounds were initially found to be poorly biodegradable, but more rapid biodegradation is observed today (3). Many atrazine-degrading bacteria have now been isolated (4–6). They invariably contain highly conserved genes encoding enzymes that hydrolitically displace substituents from the s-triazine ring carbon atoms to generate cyanuric acid (Fig. 1). The genes, trzN, atzA, atzB, and atzC, are found on plasmids and now are distributed globally (7, 8). These observations are consistent with the idea that a new metabolic pathway for atrazine catabolism may have evolved and spread in recent evolutionary times (9).

The s-triazine herbicides, such as atrazine (2-chloro-4-isopropylamino-6-ethylamino-1,3,5-triazine) and ametryn (2-thiomethyl-4-isopropylamino-6-ethylamino-1,3,5-triazine), are metabolized readily by dedicated enzymes. The enzymes have been purified to homogeneity and shown to be inactive with structurally analogous pyrimidines and other closely related compounds (10–13). Moreover, the enzymes are isolated from bacteria obtained from herbicide-contaminated environments. Bacteria that completely assimilate atrazine initiate metabolism via an initial dechlorination reaction catalyzed by atrazine chlorohydrolase. Atrazine chlorohydrolase has been used for herbicide remediation and shown to reduce atrazine levels by 90% in a contaminated soil (14). Two distinct atrazine chlorohydrolases, TrzN (4, 11) and AtzA (atrazine chlorohydrolase 1) (13), have been described. Though both enzymes likely evolved recently, their pairwise protein sequence identity of 28% suggests that they each evolved independently from two different but evolutionarily related ancestors (11). However, both TrzN and AtzA catalyze hydrolytic dechlorination reactions with atrazine and related s-triazine herbicides.

X-ray structures have been determined for chlorohydrolases that act on haloaliphatic and halobenzene substrates. Haloalkane dehalogenase, halocid dehalogenase, and 4-chlorobenzoyl-CoA dehalogenase all use an aspartate nucleophile and hydrolyze the intermediate enzyme-substrate ester to catalyze an overall hydrolytic displacement reaction (15). cis-3-Chloroacrylic acid dehalogenase proceeds via a hydratase-like mechanism (16). These structurally defined dehalogenases, which use water as a cosubstrate, are not metalloenzymes. In contrast, TrzN and AtzA each require a divalent metal ion to catalyze hydrolytic dehalogenation and thus differ mechanistically from other well-studied halohydrolases (4, 11, 13).

TrzN and AtzA are both metalloenzymes of the amidohydrolase superfamily that contain zinc(II) and iron(II), respectively (11, 13). A number of well studied amidohydrolases, such as adenosine deaminase (17) and cytosine deaminase (18), cata-
lyze hydrolytic deamination from diazine ring systems, which is somewhat comparable to hydrolytic dechlorination from an s-triazine ring substrate. Yet, AtzA has a high degree of substrate specificity, catalyzing only dehalogenation reactions (10). In contrast, TrzN has a broader substrate specificity and displaces thiomethyl and methoxy groups from the related s-triazine herbicides ametryn and atronate (2-methoxy-4-isopropylamino-6-ethylamino-1,3,5-triazine), respectively. TrzN has been demonstrated to be present in atrazine-degrading bacteria isolated from four different continents (9). TrzN has a higher \( k_{cat}/K_m \) for s-triazine herbicides than does AtzA, making it preferable for remediation of s-triazine herbicide contamination in natural and engineered environments.

Here, we investigated the structural and catalytic properties of TrzN by analyzing three crystal structures and two sets of active site mutants. The 1.4 Å crystal structure of the wild-type enzyme reveals it to be a unique member of the amidohydrolase superfamily with a four-coordinate metal center and lacking an aspartate ligand present in other proteins in the amidohydrolase superfamily (20). The aspartate in TrzN is replaced by a threonine residue that is within hydrogen-bonding distance to the zinc-bound water. In this regard, TrzN resembles carbonic anhydrase (CA).\(^2\) Crystal structures also were determined with a catalytically impaired TrzN-E241Q, containing bound ametryn or atronate, respectively. The data suggests that Glu\(^{241}\) in TrzN acts as a proton donor, a function comparable to the conserved glutamate in several other amidohydrolases. Taken together, these data provide insights into enzyme evolution of naturally occurring, substrate-specific enzymes that act to remove anthropogenic chemicals from the environment.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Growth Conditions, and Protein Purification—**Escherichia coli strain BL21(DE3)(PET28b+::trzN(pAG)) and strains containing mutant plasmids were grown and induced in LB media containing 50 mg/ml kanamycin and 30 mg/ml chloramphenicol as described previously (11) with the following modifications. Initial cultures were grown at 37 °C. When cultures reached an optical density of 0.5 at 600 nm, they were transferred to 15 °C for 30 min without shaking. Both chaperonin and TrzN expression plasmids were induced, as described previously (11).

For protein purification, all solutions contained a base buffer of 0.1 M sodium phosphate buffer, pH 7.0, with 10% glycerol. The HisTrap chelating column was washed with 60 ml buffer, followed by three washes of 30, 30, and 60 ml with 0.15, 0.18, and 0.2 M imidazole, respectively. Enzyme was eluted from the column with 30 ml of 0.5 M imidazole, which subsequently was removed from the enzyme preparation via three 4–6 h of dialysis exchanges at 4 °C. The second exchange was supplemented with 65 μM ZnSO₄.

**Chemicals and Reagents—**Atrazine, ametryn, and atronate were provided generously by Syngenta Crop Protection (Greensboro, NC). All other s-triazines used in this study were synthesized in our laboratory as described previously (19).

**Computational Methods—**All crystallographic figures were produced in Chimera (21). Sequences of the amidohydrolase superfamily were obtained from the Structure Function Linkage Database (22). Sequence clustering was performed by combining functional information with network clusters obtained from the program structureViz, used as described (23, 24). Connectivity is depicted at the specified BlastP score cut-offs.

**Crystallization and Data Collection—**Two different crystal forms of atrazine chlorohydrolase (TrzN) were grown by the hanging drop method at 25 °C: 1) wild-type TrzN with Zn\(^{2+}\) and 2) TrzN mutant E241Q with Zn\(^{2+}\). The two crystal forms were obtained using different conditions for wild-type and mutant TrzN. For the crystal form of wild-type TrzN, the protein solution contained wild-type TrzN (21 mg/ml) in 25 mM MOPS (pH 5.5) and 1 mM ZnCl\(_2\); the precipitant contained 25% PEG 3350, 0.1 M Bis-Tris (pH 5.5), 0.2 mM ammonium sulfate, and 1 mM ZnCl\(_2\). For this sample, crystals appeared in 7–8 days and exhibited diffraction consistent with the space group \(P_{2_1}2_1\), with two molecules of the protein per asymmetric unit (Table 1, column 1). Prior to data collection, the crystals were transferred for \(\sim 15\) s to a cryoprotectant solution composed of the mother liquid and 20% glycerol. For TrzN-E241Q, the protein solution contained TrzN-E241Q (6.6 mg/ml) in 25 mM MOPS (pH 5.5) and 1 mM ZnCl\(_2\), the precipitant contained 25% PEG 3350, 0.1 M Bis-Tris (pH 5.5), 0.2 mM ammonium sulfate, and 1 mM ZnCl\(_2\). For this sample, crystals appeared in 2 weeks and exhibited a diffraction pattern consistent with space group \(P_{2_1}2_1\), with two molecules of the protein per asymmetric unit.

The TrzN substrates atronate and ametryn are relatively insoluble. Therefore, powders of these compounds were used for soaking experiments. The crystal containing TrzN-E241Q was soaked for 1 h in cryobuffer composed of its mother liquid, 15% glycerol, and excess of the atronate powder (Table 1, column 2). Another crystal containing TrzN-E241Q was soaked for 4 h in cryobuffer composed of its mother liquid, 15% glycerol, and excess of the ametryn powder (Table 1, column 3).

After incubations in their corresponding cryobuffers, the crystals of all three TrzN crystal forms (Table 1) were flash-cooled in a nitrogen stream. All 3 x-ray diffraction data sets were collected at the NSLS X4A beamline (Brookhaven National Laboratory) on an Area Detection Systems Corporation (Posway, CA) charge-coupled device detector. Diffraction intensities were integrated and scaled with programs DENZO.
and SCALEPACK (25). The data collection statistics are given in Table 1.

**Structure Determination and Model Refinement**—The structures of all three TrzN crystal forms (Table 1, columns 1–3) were solved by molecular replacement, using the fully automated molecular replacement pipeline BALBES (26), using only input diffraction and sequence data. The protein part of putative cytosine deaminase (Protein Data Bank code 2PAJ) was used by BALBES as a template in all three structure determinations. Partially refined structures of all three TrzN forms (Table 1, columns 1–3) were the outputs from BALBES without any manual intervention. Subsequently, several iterative cycles of refinement were performed for each crystal form, including the model rebuilding with COOT (27), refinement with PHENIX (28), and automatic model rebuilding with ARP (29). Crystallographic refinement statistics for all determined TrzN structures are provided in Table 1.

The model of the wild-type TrzN was refined at 1.4 Å with an $R_{cryst}$ of 0.168 and an $R_{free}$ of 0.189. The final structure contained protein residues 1–453 and one well defined Zn$^{2+}$ ion in each monomer of the dimer.

The model of the TrzN-E241Q-atratone complex was refined at 1.64 Å with an $R_{cryst}$ of 0.189 and an $R_{free}$ of 0.214. The final structure contained protein residues 1–453 and one well defined Zn$^{2+}$ ion in each monomer of the dimer. The first monomer of the dimer contained a well defined atratone molecule bound in the active site. The electron density for ametryn was weaker than for atrazine molecule in the previous structure. Final crystallographic refinement statistics for the three TrzN structures are listed in Table 1.

**Protein Concentration, Enzyme Assays, and Steady-state Kinetic Determinations**—Standard protein concentrations were determined with the Protein Assay Dye Reagent in accordance with manufacturer’s instructions (Bio-Rad). Activity assays for atrazine and ametryn were conducted as described previously (11), whereas activity assays for atratone were analyzed via high pressure liquid chromatography as described previously (12). Kinetic constants were calculated in 0.1 M sodium phosphate buffer, pH 7.0, from initial hydrolysis rates at 7–10 different substrate concentrations, ranging from 1.5–150 mM. All samples were done in triplicate. Control samples without enzyme were analyzed in parallel, and no spontaneous activity was observed. Apparent $K_m$ and $V_{max}$ values were estimated by nonlinear regression according to the Michaelis-Menten equation: $v = \frac{(V_{max} \times [S])}{(K_m + [S])}$, using GraphPad Prism software (version 5). Steady-state rate measurements were determined at different pH values as described above with 150 mM atrazine or ametryn in the following 100 mM buffers: sodium acetate (pH 4 and 5), sodium phosphate (pH 6, 7, and 8), borate (pH 9), and CAPS (pH 10 and 11).

**Site-directed Mutagenesis**—The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to make all mutants. The following primers were used to construct the mutants: T325S, 5’-GGTGTGCTGGCCGGACCCGCTTCCAGCC-3’ and 5’-GCTGCGCCGGAGGCCTTCCAGCC-3’; T325D, 5’-GGTGTGCTGGCCGGACCCGCTTCCAGCC-3’ and 5’-GCTGCGCCGGACCCGCTTCCAGCC-3’; T325E, 5’-GGTGTGCTGGCCGGACCCGCTTCCAGCC-3’ and 5’-GCTGCGCCGGACCCGCTTCCAGCC-3’; E241K, 5’-GGTGTGCTGGCCGGACCCGCTTCCAGCC-3’ and 5’-GCTGCGCCGGACCCGCTTCCAGCC-3’; E241Q, 5’-GGTGTGCTGGCCGGACCCGCTTCCAGCC-3’ and 5’-GCTGCGCCGGACCCGCTTCCAGCC-3’.

| Data collection | WT TrzN-Zn$^{2+}$ | TrzN-E241Q-Zn$^{2+}$-atratone | TrzN-E241Q-Zn$^{2+}$-ametryn |
|-----------------|-------------------|-------------------------------|-----------------------------|
| Space group     | $P_2_1$           | $P_2_1$                       | $P_2_1$                     |
| No. of mol. in asymmetrical units | 2                 | 2                             | 2                           |
| Cell dimensions | $a, b, c$ (Å)     | 57.55, 102.62, 80.55          | 57.38, 101.62, 80.77        | 57.40, 100.84, 80.63         |
| $\beta$         | 104.75°           | 104.45°                       | 104.42°                     |
| Resolution (Å)  | 1.40              | 1.64                          | 1.93                        |
| No. of unique reflections | 176,330           | 108,286                       | 65,334                      |
| $R_{merge}$     | 0.083             | 0.071                         | 0.066                       |
| $I/\sigma(I)$   | 19.9              | 22.3                          | 24.1                        |
| Completeness (%)| 99.6              | 98.6                          | 98.4                        |

$^a$ r.m.s.d., root mean square deviation.

$^b$ PDB, Protein Data Bank.

### Table 1

Data collection and refinement statistics for crystals of the atrazine chlorohydrolase TrzN from *Arthrobacter aurescens* TC1

| Data collection | WT TrzN-Zn$^{2+}$ | TrzN-E241Q-Zn$^{2+}$-atratone | TrzN-E241Q-Zn$^{2+}$-ametryn |
|-----------------|-------------------|-------------------------------|-----------------------------|
| Space group     | $P_2_1$           | $P_2_1$                       | $P_2_1$                     |
| No. of mol. in asymmetrical units | 2                 | 2                             | 2                           |
| Cell dimensions | $a, b, c$ (Å)     | 57.55, 102.62, 80.55          | 57.38, 101.62, 80.77        | 57.40, 100.84, 80.63         |
| $\beta$         | 104.75°           | 104.45°                       | 104.42°                     |
| Resolution (Å)  | 1.40              | 1.64                          | 1.93                        |
| No. of unique reflections | 176,330           | 108,286                       | 65,334                      |
| $R_{merge}$     | 0.083             | 0.071                         | 0.066                       |
| $I/\sigma(I)$   | 19.9              | 22.3                          | 24.1                        |
| Completeness (%)| 99.6              | 98.6                          | 98.4                        |

$^a$ r.m.s.d., root mean square deviation.

$^b$ PDB, Protein Data Bank.
GTGGTAGAAGTGCTGGGAGGC-3′; and Y67F, 5′-CACCAGCACCTGTTCAAGGGCATG-3′ and 5′-CAT-TGGCCCTTGAAACAGGTGCTGGGTG-3′.

RESULTS AND DISCUSSION

Evolutionary Linkage of TrzN to Other Amidohydrolase Superfamily Members—Proteins in the amidohydrolase superfamily almost invariably contain one or two catalytically essential divalent metal atoms per enzyme subunit (20). Using a network clustering of amino acid sequences with a cutoff of e−5, TrzN clustered with the monometallic amidohydrolases that have evolved their function recently (11). As shown in Fig. 2B, the closest relative to TrzN is AtzB, hydroxyatrazine N-ethylamidohydrolase. However, these two proteins only share 31% sequence identity, demonstrating that TrzN is quite divergent from all other characterized amidohydrolases. The proteins represented by the light blue circles are proteins of unknown function. However, Tm0936 (accession no. 1P1M_A) from Thermotoga maritima is one protein in that cluster with a known function and catalyzes the deamination of 5-methylthioadenosine, S-adenosyl-L-homocysteine, and adenosine (30). This adenosine deaminase is thought to have evolved independently and not directly from the cluster of adenosine deaminases shown in Fig. 2A. This clustering (shown in light blue) represents proteins most closely related to the s-triazine metabolizing enzymes and may represent a lobe of the amidohydrolase superfamily with great functional plasticity.

Crystal Structure of Wild-type TrzN—Solving the TrzN crystal structure to 1.4 Å resolution (Fig. 3) allowed a more detailed comparison with other closely related amidohydrolase superfamily members. Though reported to be monomeric in solution (11), TrzN crystallized as a dimer. The difference in oligomeric configuration may be due to pH and ionic strength differences between crystallization conditions and those used for gel-filtration studies. The dimer consists of two separate subunits that interact along one side of the barrel. The loop and α helix region from residues 408–432, an α helix from residues 348–363, and a loop from residues 85–94 together comprise much of the dimer interface. The active sites of both subunits are accessible in the dimeric form. The N-terminal 54 amino acid residues make up two β sheets that are external to the barrel and, when looking down the axis of the βαβ barrel, are located behind the floor of the barrel (Fig. 3, magenta).

Monometallic superfamily members often have three histidines and an aspartate residue as metal ligands scattered in conserved locations throughout the linear sequence of the protein (Fig. 4A). In those proteins, water serves as a fifth metal ligand. A fourth histidine serves as a base and interacts with a metal-coordinated water or hydroxide ion to facilitate nucleophilic attack on the substrate (31). The linear sequence of TrzN has the four conserved histidine residues but lacks the conserved aspartate that typically serves as a metal ligand (Fig. 4A). A

FIGURE 2. Clustering network diagram of a subset of amidohydrolase superfamily proteins. Nodes represent protein sequences, and edges show the degree of sequence relatedness as determined by BlastP at a specified e-score cut-off. A, amidohydrolase protein network using a cut-off score of 1e−5. Sea green nodes represent dimetallic amidohydrolase proteins such as ureases and O-aminoacylases. Red represents distant monometallic superfamily members. Monometallic superfamily members in closer proximity to TrzN are colored as indicated: GUDAs are green, Structure-Function Linkage Database group no. 63 of unknown function are light blue (unknown63), TrzN is yellow, AtzA is light green, AtzB is magenta, an AtzB homolog of unknown function is orange, Tm0936 from T. maritima is purple, TriA is dark blue, and TrzA is pink. B, proteins in the rectangle of A were reanalyzed with a cut-off score of 1e−38.

FIGURE 3. TrzN dimer structure in stereo. A dimer is shown with subunit A colored cyan and blue and subunit B colored red and magenta. The histidine metal ligands are displayed in yellow to denote the position of the active site.
threonine residue (Thr^{325}) in TrzN aligns with the conserved aspartate. Superposition of the TrzN structure with those of three structurally defined amidohydrolase superfamilies showed that the functional atoms in the histidine metal ligands are nearly superimposable (Fig. 4B). Although the threonine is in the same region as the corresponding aspartic residues in other superfamily members (Fig. 4B), the threonine oxygen is 3.3 Å from the zinc and is too distant to be a metal ligand. This compares to an Asp-metal distance of 2.3–2.5 Å in the other amidohydrolases shown in Table 2. Thus, TrzN has a different metal coordination environment than other amidohydrolases. Typically, the mononuclear metal centers in the amidohydrolases are five-coordinate, whereas in TrzN, the metal is four-coordinate. Angles between metal-water-histidine ligands His^63, His^65, and His^233 are 108.8, 120.4, and 107.6°, respectively. The histidine ligand-zinc-histidine ligand angles range from 100.6–115.5°. This geometry forms an irregular tetrahedron. Table 2 compares active site distances in TrzN with some of the most well-studied mononuclear amidohydrolase superfamily members: adenosine deaminase (ADDA, Protein Data Bank code 1A4M), cytosine deaminase (CYDA, Protein Data Bank code 1K70), guanine deaminase (GUDA, Protein Data Bank code 2UZ9), and Tm0936, a protein crystallized from a structural genomics project from Thermatoga maritima (2PLM). The distances in TrzN shown in Table 2 are within the range of other amidohydrolase members with the exception of Thr^{325}.

**Mutation of Thr^{325}**—The side chain oxygen atom of Thr^{325} is 2.6 Å from the oxygen atom of the water (or hydroxide) that is coordinated to the zinc atom. This suggested that this residue could be assisting in positioning the water for nucleophilic attack at C-2 of the s-triazine ring. Site-directed mutants were constructed to further investigate...
the function of Thr^{325}. Relative to the wild-type, the conservative mutation T325S showed a 12-fold reduction in \( k_{cat}/K_m \) with atrazine as a substrate, due to a 17-fold decrease in \( k_{cat} \) and a 1.4-fold reduction in \( K_m \) (Table 3). Kinetic values with ametryn as the substrate showed a similar trend, with a 26-fold decrease in \( k_{cat}/K_m \), due to a 13-fold decrease in \( k_{cat} \) and a 2-fold increase in \( K_m \) (Table 3). Mutagenesis of Thr^{325} to aspartate or glutamate resulted in a TrzN with no detectable activity and thus greatly diminishing activity (33). Comparison of the Glu241 in TrzN aligns with a conserved glutamate in other amidohydrolase superfamily members, which is involved in metal ligation.

The positioning and apparent functioning of Thr^{325} in TrzN has a known precedent in carbonic anhydrase (CA), a protein that is in a different structural family but was observed here to have a similar active site (32–36). The overall folds differ markedly. Whereas TrzN consists of an \((\beta\alpha)_8\) barrel, CA has an ellipsoidal shape with a deep funnel-shaped active site cavity. Therefore, the two proteins have different structural folds. Despite their marked structural differences, the active sites show nearly the same arrangement of residues contributing ligands to the respective zinc atoms (Fig. 5). The arrows in Fig. 5 highlight the histidine nitrogens that coordinate each zinc atom. In the middle histidine of the sequence, CA uses the N-δ of the histidine ring for metal ligation, whereas TrzN uses the N-ε, yet the respective position of the liganding nitrogens are conserved. Similarly, the threonine oxygen is located 2.6 and 2.8 Å from the metal-bound water (hydroxide) in TrzN and CA, respectively. Given the complete lack of sequence and structural relatedness of TrzN and CA, this suggests convergent evolution of disparate enzymes. Furthermore, CA mutants had similar affects on kinetic parameters as did the TrzN mutants analyzed here. The T199S CA mutation resulted in a 4-fold reduction in \( k_{cat}/K_m \) (35), whereas aspartate and glutamate mutations resulted in decreased activity of approximately four orders of magnitude (36). Crystal structures of the CA T199D and T199E mutants show that the residues with a carboxylate group formed a metal ligand, displacing zinc bound hydroxide, and thus greatly diminishing activity (33). Comparison of the mechanism of TrzN and CA is discussed further below.

**TABLE 2**
Comparison of amidohydrolase superfamily member structural distances

| Table 2 |  |  |  |  |  |
|---|---|---|---|---|---|
| Addenosine deaminase (ADDA) | Cytosine deaminase (CYDA) | Guanine deaminase (GUDA) | Thermatoga maritima protein (Tm0936) | TrzN |
| Metal ligands | Zinc | Zinc | Zinc | Zinc |
| Metal to water \(^{\alpha}\) | 2.2–2.5 | 3.7 | 3.8 | 4.1 |
| Asp ligand/Thr to metal | 2.4 | 2.5 | 2.3 | 3.3 |
| His (water-activating) to metal | 4.2 | 3.7 | 3.8 | 4.1 |
| Metal | Zinc | Zinc | Zinc | Zinc |
| Metal to water \(^{\beta}\) | 2.1 | 2.0 | 3.0 | 2.0 |
| Asp ligand/Thr to water \(^{\alpha}\) | 2.8 | 2.9 | 3.4 | 2.7 |
| His (water-activating) to water \(^{\beta}\) | 3.2 | 2.8 | 3.3 | 2.6 |

\(^{\alpha}\) The appropriate oxygens in the inhibitor 6-hydroxy-1,6-dihydro purine nucleoside, transition state analog 4-hydroxy-3,4-dihydro-1H-pyrimidin-2-one and product xanthine were used in the cases of adenosine, cytosine, and guanine deaminases, respectively, as these compounds replace the active site water.

**TABLE 3**
Kinetic constants for wild-type and mutant TrzN with atrazine as a substrate

| Table 3 |  |  |  |  |  |
|---|---|---|---|---|---|
| Atrazine | Ametryn |  |
| \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) |
| Wild-type | 1.49 ± 0.02 | 19 ± 1 | 78 ± 5 | 6.04 ± 0.05 | 3.9 ± 0.2 | 1530 ± 90 |
| T325S | 0.0869 ± 0.0008 | 133 ± 0.6 | 63 ± 0.3 | 0.467 ± 0.0003 | 7.8 ± 0.3 | 60 ± 2 |
| E241Q | 0.137 ± 0.002 | 3.1 ± 0.3 | 44 ± 5 | ND | ND | ND |

\(^{\alpha}\) ND, steady-state kinetic values could not be determined. Long incubations indicated specific activity of 0.0006 μmol/min/mg of protein.
In the present study, a TrzN-E241Q mutant was slightly impaired with atrazine as a substrate and greatly impaired with ametryn and atratone (Table 3). Rates of hydrolysis with ametryn or atratone as substrates were too low to obtain kinetic constants. With atrazine as a substrate, $k_{cat}/K_m$ was reduced only 1.8-fold, due to an 11-fold decrease in $k_{cat}$ and a concomitant 6-fold decrease in $K_m$ (Table 3). In adenosine deaminase, the E217Q mutation produced mainly $k_{cat}$ affects, with a 3–4 order of magnitude decrease in $k_{cat}/K_m$ (37).

Due to these differences, the pH profiles of wild-type and E241Q mutant proteins with the substrate atrazine also were examined (Fig. 6). Activity with the wild-type enzyme showed a pH optimum at 7.0 that decreased to near baseline levels at pH 10. The E241Q mutant showed a broad plateau of activity with atrazine from pH 6 to 10. These data, in conjunction with the crystal structures of bound substrates discussed below, are consistent with the structural placement of Glu241 near N-1 of atrazine and for Glu241 to be protonated at neutral pH. The downward limb of the pH profile suggested that glutamate is being titrated at higher pH and has an abnormally high pK_a in the vicinity of 8.5.

The crystal structures of the E241Q mutant bound with ametryn and atratone were solved at 1.93 and 1.64 Å, respectively (Fig. 7, A and B). The backbone tracings of TrzN with the addition of ametryn and atratone were not demonstrably different from that of the native enzyme. Furthermore, the placement of ametryn and atratone in the active site was virtually identical in each structure (Fig. 7 C). The plane of the s-triazine ring in TrzN is similar to that of other amidohydrolase superfamily members with respective ring compounds bound, as indicated, by backbone-aligned structures with guanine deaminase (Protein Data Bank code 2UZ9), cytosine deaminase (Protein Data Bank code 1K70), and adenosine deaminase (Protein Data Bank code 1A4M). Placement of the ametryn and atratone in the active site also agreed with initial docking studies with only slight differences in the conformation of the alkyl groups (data not shown). Note that a published homology modeled structure of AtzA showed the alkyl groups in the opposite orientation with respect to the conserved active-site glutamate (40). This conformation is not likely as one of the N-alkyl binding pockets is clearly smaller than the other. All evidence suggests that the N-ethyl group docks in the smaller pocket preferentially. This discrepancy in the model could be due to the fact that only a portion of the protein was modeled and not the entire structure.

The distance from the catalytic water to the reactive carbon is 2.7 Å for ametryn and 2.8 Å for atratone, consistent with the nucleophilic
substitution reaction of the amidohydrolase superfamily. The conserved superfamily placement of a glutamate in a position to potentially donate a proton to nitrogen-1 of the s-triazine ring also is observed (3.3 Å from O-e on residue 241 to N-1 of either ametryn or atratone). The other e position is located 3.4 Å from the nitrogen on the N-ethyl side chain. With the proton added to the wild-type enzyme in preliminary docking studies, the amino hydrogen of the N-ethyl side chain is 2.5 Å from the carbonyl oxygen of E241, thereby potentially contributing electrostatic interactions. These data and kinetic data discussed above suggest a multidimensional contribution of Glu241 to substrate binding. The proximity of the E241Q side chain carbonyl oxygen to the protonated nitrogen on the N-ethyl side chain, discussed above, suggests that this residue also may contribute to substrate binding as well.

The position of the leaving groups in ametryn and atratone are similar, with the leaving group sulfur/oxygen atom positions differing by 0.5 Å and the methyl group carbon atoms differing by 0.7 Å with respect to each other. The large differences in reactivity of ametryn and atratone are thus likely due to differences differing by 0.5 Å and the methyl group carbon atoms are similar, with the leaving group sulfur/oxygen atom positions.

The enzyme surface into which the leaving groups are displaced contains a mixture of hydrophobic and polar characteristics. Residues that line the leaving group pocket include Trp305, Pro299, Met303, Ser329, Thr325, His274, Gln241, and Trp85. This first tier of residues are within 5 Å of the leaving group. Additional residues that fully enclose the leaving group at a distance of 5–10 Å are Leu296, Arg302, Met82, and Asp300 (with the hydrophobic part; the carboxylic acid group is pointing away from the leaving group).

The position of the two N-alkyl side chains in both the ametryn and atratone E241Q mutant proteins are nearly identical. The N-ethyl side chain of both substrates are contained within a surface created by Tyr215, Leu86, Cys211, His238, Gln241, and Met82. The pocket is snug with residues within 3.3–4.0 Å of the side chain. There is, however, a channel occupied by a set of three waters (57.5, 102.9, and 112.5), which has the first water residing 4.6 Å from the methylene carbon of the ethyl group, which could potentially house a longer side chain. This may be how TrzN catalyzes reactions with symmetrical substrates containing longer and more branched side chains at both N-alkyl positions, albeit at reduced rates (10). The N-isopropyl binding pocket is slightly larger. An O-e from Glu68 is 3.0 Å from the nitrogen in the side chain, therefore potentially contributing hydrogen bond interactions to binding. The other residues that line the N-isopropyl binding pocket include His65, Tyr67, Leu131, Val89, and Trp85. The two N-alkyl binding pockets have some similarity. Both contain glutamate, histidine, tyrosine, and leucine residues with the glutamate residues in relatively close proximity to the nitrogen of the side chains.

**Mechanism and Comparison with Carbonic Anhydrase—**

The overall structure of TrzN is consistent with those of other amidohydrolase superfamily members. However, the data obtained here indicated that TrzN shares some catalytic features with the amidohydrolases and some with the carbonic anhydrases. First, unlike most monometallic amidohydrolases that have a five-coordinate metal, the zinc in TrzN is four-coordinate (Fig. 8A). The native enzyme contains three histidine ligands and a water or hydroxide oxygen ligand.

The threonine in TrzN is unique and substitutes for an aspartate that is present in 1,749 amidohydrolase superfamily members. We have examined. In some amidohydrolase proteins, the aspartate is thought to serve as a metal ligand; in others, it is somewhat too distant for direct metal ligation. The threonine oxygen in TrzN is too distant (3.3 Å) from the zinc atom to be a ligand, but it is positioned such that the oxygen could bond to a hydrogen atom on the zinc bound hydroxide or water. The distance and positioning are remarkably reminiscent of carbonic anhydrase (Figs. 5 and 8). In TrzN, it is likely that His211 also interacts with the zinc-bound water. Superimposed on this, glutamate 241 serves as a proton donor to the s-triazine ring, facilitating nucleophilic aromatic substitution. It is logical to assume that ring protonation provides significant catalytic enhancement with substrates containing an S-methyl or O-methyl leaving group. With a chloride leaving group, the reaction may be sufficiently facile that catalysis proceeds reasonably efficiently without ring protonation.

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