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TrzN from *Arthrobacter aurescens* TC1 Is a Zinc Amidohydrolase

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TrzN, the broad-specificity triazine hydrolase from *Arthrobacter* and *Nocardioides* spp., is reportedly in the amidohydrolase superfamily of metalloenzymes, but previous studies suggested that a metal was not required for activity. To help resolve that conundrum, a double chaperone expression system was used to produce multimilligram quantities of functionally folded, recombinant TrzN. The TrzN obtained from *Escherichia coli* (*trzN*) cells cultured with increasing zinc in the growth medium showed corresponding increases in specific activity, and enzyme obtained from cells grown with 500 μM zinc showed maximum activity. Recombinant TrzN contained 1 mole of Zn per mole of TrzN subunit. Maximally active TrzN was not affected by supplementation with most metals nor by EDTA, consistent with previous observations (E. Topp, W. M. Mulbry, H. Zhu, S. M. Nour, and D. Cuppels, Appl. Environ. Microbiol. 66:3134–3141, 2000) which had led to the conclusion that TrzN is not a metalloenzyme. Fully active native TrzN showed a loss of greater than 90% of enzyme activity and bound zinc when treated with the metal chelator 8-hydroxyquinoline-5-sulfonic acid. While exogenously added zinc or cobalt restored activity to metal-depleted TrzN, cobalt supported lower activity than did zinc. Iron, manganese, nickel, and copper did not support TrzN activity. Both Zn- and Co-TrzN showed different relative activities with different *s*-triazine substrates. Co-TrzN showed a visible absorption spectrum characteristic of other members of the amidohydrolase superfamily replaced with cobalt.

Bacteria metabolize *s*-triazine herbicides via an initial hydrolytic displacement of chloride (atrazine and simazine), S-alkyl (ametryn and prometryn), or O-methyl (atratone) substituents (2, 15, 21, 22). Gram-positive triazine-metabolizing bacteria typically initiate metabolism via the enzyme TrzN, while gram-negative bacteria express AtzA (Fig. 1). TrzN from *Arthrobacter aurescens* TC1 shows 26% amino acid sequence identity to AtzA from *Pseudomonas* sp. strain ADP. Although both AtzA and TrzN catalyze halide displacement, only TrzN has been demonstrated to displace S-methyl, O-methyl, and other substituents (Fig. 1). TrzN has been purified by two different research groups, and its basic kinetic and substrate specificity parameters have been investigated (20, 22). TrzN is proposed to be a member of the amidohydrolase superfamily (5), a large protein superfamily which contains members that require a mononuclear or binuclear metal center for enzyme activity (3). A recent review defines characteristics of the superfamily in the context of 16 X-ray structures determined for member proteins (18). Fifteen were observed to contain one or more transition metal atoms, and the metal is catalytically relevant in those amidohydrolases in which this property has been studied.

In this context, it was surprising that the only study investigating the role of metals in TrzN activity suggested that it was not a metalloenzyme (22). In that study, TrzN from *Nocardioides* sp. was purified to homogeneity and the addition of iron, zinc, cobalt, or copper to purified TrzN was reported not to increase enzyme activity. Moreover, adding the metal chelator EDTA to reaction mixtures did not inhibit enzyme activity. Metal determination experiments were not possible because only 80 μg of protein was purified to homogeneity from the native organism (22). In an effort to obtain more protein, the *trzN* gene was cloned into *Escherichia coli* (5). This allowed the gene to be sequenced, but the recombinant *E. coli* cells did not express active TrzN (5). Subsequently, active TrzN was expressed in a recombinant host by using a bacterial artificial chromosomal vector, but this provided only a small amount of enzyme (12). More recently, TrzN expression was increased, but enzyme activity remained low (20). In that study, most of the TrzN enzyme expressed was found in inclusion bodies and largely inactive. This rendered studies of metal content and activation difficult to interpret.

To overcome these problems, we describe here the use of a double chaperone system which provides consistent folding of TrzN in vivo and the ability to produce TrzN in high yields and activity. This allowed metal determination, chelation, and reconstitution experiments establishing that native TrzN contains a mononuclear zinc center essential for enzyme activity. Zinc could be removed and replaced with cobalt, but not other metals, and yielded active TrzN. Zn- and Co-substituted TrzN showed different relative activities with different substrates, an observation that could be relevant to the metabolism of herbicides in nature.

MATERIALS AND METHODS

Chemicals. All buffers and routine biochemicals were obtained from Sigma-Aldrich and were of the highest purity available. The following compounds were graciously provided by Syngenta (Greensboro, N.C.): atrazine, simazine, terbutylazine, and ametryn. Simetryn was obtained from Sigma-Aldrich. All other compounds were synthesized in our laboratory. Cyanoatrazine was synthesized as

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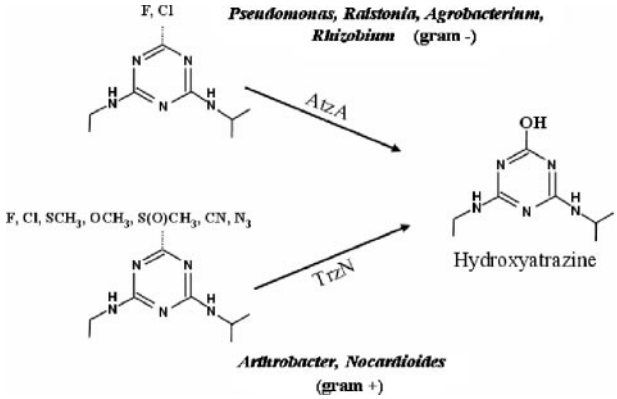

FIG. 1. Different substituted *s*-triazines can be hydrolyzed by AtzA and TrzN to produce hydroxyatrazine. AtzA is known to be active only with fluoride or chloride, but many other substituents are displaced by TrzN.

previously described (15), and thioatrazine was prepared by refluxing atrazine, thiourea, and water in ethanol under a nitrogen atmosphere as previously described (20).

Expression and purification of TrzN. Plasmid pAG, containing the chaperones *groEL* and *groES* (6), was transformed into *E. coli* BRL21(DE3) containing plasmid pET28b+::*trzN* (20). Strain BRL21(DE3)(pET28b+::*trzN*) (pAG) was grown in LB medium (13) containing kanamycin (50 μg/ml) and chloramphenicol (30 μg/ml) at 15°C, with shaking at 150 rpm. When cultures reached an optical density of 0.5 at 600 nm, the chaperones were induced by the addition of 0.0015% (wt/vol) L-arabinose, and 1.5 μM IPTG (isopropyl-β-D-thiogalactopyranoside) was added after an additional 90 min of incubation at 15°C. Induced cells were grown for an additional 16 h under the same conditions, cultures were centrifuged at 10,000 × *g* for 10 min at 4°C and washed three times with 0.85% (wt/vol) NaCl, and cell pellets were resuspended in 30 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 10% glycerol. Cells were broken by passage, three times, through a chilled French pressure cell operated at 140 MPa, and cell extracts were obtained by centrifugation at 18,000 × *g* for 90 min at 4°C. Lysates were applied to a 5-ml HisTrap chelating column (Amersham Pharmacia Biotech, Piscataway, NJ), complexed with Ni^{2+}, according to the manufacturer's instructions. The column was washed with 15 ml 0.1 M sodium phosphate buffer, pH 7.0, followed by two washes with the same buffer supplemented with 0.1 M and 0.25 M imidazole, respectively. All buffers contained 10% glycerol. Enzyme was eluted from the column with 15 ml of 0.5 M imidazole in 0.1 M sodium phosphate buffer, pH 7.0, and the purified enzyme was concentrated using a Centricon-30 filtration unit (Amicon, Beverly, MA). Imidazole was removed from the enzyme preparation by dialysis, twice, at 4°C for 4 h against 4 liters of 0.1 M sodium phosphate buffer, pH 7.0, containing 10% glycerol. Enzyme purity and subunit molecular weight were estimated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (13).

Enzyme assay. Enzyme activity was measured by monitoring the disappearance of the substrate ametryn at 264 nm by using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). The product, hydroxyatrazine, had negligible absorbance at this wavelength. Reactions (1 ml) were carried out at 37°C in 0.1 M sodium phosphate buffer, pH 8.0, containing 132 μM ametryn. Reactions were initiated by the addition of enzyme, and the molar absorbance at 264 nm for ametryn under these conditions was determined to be 5 mM^{-1} cm^{-1}. Enzyme activity against other *s*-triazines was determined using the same procedure, with the following changes in wavelengths and molar absorbance (ε) values used for measuring and calculating substrate disappearance: atrazine, λ = 264 nm, ε = 3.5 mM^{-1} cm^{-1}; dipropetryn, λ = 255 nm, ε = 7.5 mM^{-1} cm^{-1}; simetryn, λ = 264 nm, ε = 5.1 mM^{-1} cm^{-1}; terbutylazine, λ = 264 nm, ε = 2.7 mM^{-1} cm^{-1}; simazine, λ = 264 nm, ε = 3.1 mM^{-1} cm^{-1}; and cyanoatrazine, λ = 300 nm, ε = 2.1 mM^{-1} cm^{-1}.

Gel filtration chromatography. The holoenzyme molecular weight was estimated by gel filtration chromatography on a Superose 12 HR column using a fast protein liquid chromatograph system (Pharmacia, Uppsala, Sweden). The column was equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.15 M KCl, at a flow rate of 0.3 ml min^{-1}. The column was calibrated with proteins and compounds of known molecular weight: thyroglobulin, 670,000; gamma globulin, 158,000; chicken ovalbumin, 44,000; horse myoglobin, 17,000; and vitamin B_{12}, 1,350.

Influence of growth medium Zn^{2+} concentration on TrzN activity. The effect of Zn^{2+} concentration in the growth medium on TrzN activity and enzyme metal content was evaluated by supplementing LB medium with 0, 20, 100, or 500 μM $ZnSO_4$. Cells were grown and the enzyme purified as described above. The specific activity of TrzN, using ametryn as substrate, was calculated as described above, and the metal concentration in enzyme preparations was determined by using inductively coupled plasma emission spectroscopy. For metal analyses, purified TrzN (2.5 mg) was dissolved in 5 ml 0.1 M sodium phosphate buffer acidified with 10% (wt/vol) HCl. Samples were incubated overnight at 90°C, and metal content was determined by inductively coupled plasma emission spectroscopy analysis at the University of Minnesota Soils Analytical Laboratory (St. Paul, MN).

Effect of chelators on TrzN activity. Purified TrzN (25 μM) was incubated at 25°C with 5 mM of 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, or EDTA, and TrzN activity was measured with ametryn as substrate, over time, as described above.

Metal-depleted enzyme and metal reconstitution. Metal-depleted TrzN was prepared by incubating purified enzyme with 5 mM 8-hydroxyquinoline-5-sulfonic acid for 8 h at 25°C. Protein was separated from free or metal-bound 8-hydroxyquinoline-5-sulfonic acid by passage through a Sephadex G-25 column (1.5 by 30 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0. Metal-depleted enzyme eluted from the column was concentrated using a Centriprep YM-30 filtration unit (Amicon, Beverly, MA). Holoenzyme (25 μM) and metal-depleted enzyme (70 μM) were incubated in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.2 mM $ZnSO_4$, $CuCl_2$, $FeSO_4$, $MnSO_4$, $CoCl_2$, or $NiCl_2$ for 30 min at 25°C. Enzyme activity was measured as described above using ametryn as substrate. Reaction mixtures were supplemented with 1.5 mM dithiothreitol to maintain iron as Fe(II). Enzyme activity was reported as the percentage of the initial activity.

The Co(II)- and Zn(II)-reconstituted TrzN protein was prepared by incubating 35 μM of metal-depleted enzyme with 250 μM of $CoCl_2$ or $ZnSO_4$. At selected time points, the activities of the reconstituted enzymes were measured using ametryn. In addition, the specific activity of the enzymes, following 2 h of reconstitution with metals, was determined using ametryn, atrazine, dipropetryn, and cyanoatrazine as substrates as described above.

Electronic spectra of Co(II)-reconstituted enzyme. Approximately 400 μM of metal-depleted TrzN was incubated for 30 min at 25°C with 300 μM $CoCl_2$ (0.75 equivalents per TrzN subunit). Visible spectra (300 to 800 nm) of samples were acquired using a Beckman DU 640 spectrophotometer (Beckman Instruments, Fullerton, CA). The calculated extinction coefficient for Co(II)-reconstituted enzyme was based on moles of enzyme subunit. Controls contained metal-depleted enzyme alone, buffer alone, and buffer plus $CoCl_2$. The influence of the competitive inhibitor thioatrazine on metal-enzyme spectra was determined as described above with 30 μM thioatrazine added.

Kinetic parameters for selected *s*-triazine herbicides. Solutions of different *s*-triazine herbicides were prepared in 0.1 M sodium phosphate buffer, pH 8.0. The compounds were incubated with purified TrzN, and changes in absorbance were recorded. The wavelengths used were as described above. Kinetic parameters were calculated from initial hydrolysis rates at different concentrations of substrate. Control samples without enzyme were analyzed in parallel, and no spontaneous activity was detected. Kinetic parameters of TrzN were calculated using the Hanes-Woolf equation: $[S]/V_0 = [S]/V_{max} + K_m/V_{max}$ (17). Linear regression of the plot $[S]/V_0$ versus [S] was used to determine the V_{max} and K_m parameters, and k_{cat} was calculated by dividing V_{max} by the moles of TrzN subunits present. For steady-state kinetic determinations, substrate concentrations ranged from 1.3 μM to 140 μM.

RESULTS

Sequence comparison of TrzN with amidohydrolase superfamily proteins. Modest sequence identity was observed in comparisons of TrzN (accession no. AAS20185) with AtzA (accession no. NP_862474), AtzB (accession no. P95442), AtzC (accession no. O52063), and *E. coli* cytosine deaminase (CodA) (accession no. P25524) (Fig. 2). The Atz proteins have been established to be members of the amidohydrolase superfamily (11, 16). This superfamily contains a number of metalloenzymes with known X-ray structures (18), of which cyto-

		% Identity
TrzN	^{57}PGLINSHQH ..... ^{232}DVRLHTH	100
AtzB	^{68}PGLINTHHH ..... ^{239}GVSLHTH	30
AtzA	^{60}PGFINAHTH ..... ^{237}AVMWTLH	27
AtzC	^{54}PGFVDAHTH ..... ^{211}DVDIDYH	19
CodA	^{56}PPFVEPHIH ..... ^{209}DRLIDVH	20

FIG. 2. Multiple sequence alignment of TrzN, AtzB, AtzA, AtzC, and CodA showing residues near known histidine ligands to the mononuclear metal center of *E. coli* cytosine deaminase (CodA). The numbers at the right signify the percent sequence identity of each protein in a pairwise alignment with TrzN.

sine deaminase is the most closely related to the atrazine enzymes in sequence alignments. The alignments suggest the presence of three conserved histidine residues known to provide ligands to a metal in cytosine deaminase (4). The remaining metal ligand(s) is more ambiguous. However, the presence of at least three ligands and the overall sequence relatedness suggested that TrzN may be a metalloenzyme. To investigate this more fully and to determine the identity of the putative metal and its stoichiometry, significant quantities of high-activity TrzN were needed. This, in turn, required the use of a highly efficient protein expression system, and that is described below.

TrzN expression, purification, and general characterization. TrzN activity was low in initial clones containing plasmid pET28b+::*trzN* (20). Subsequent transformation of plasmid pAG (6) expressing the chaperone proteins GroEL and GroES into *E. coli* (pET28b+::*trzN*) yielded cells expressing significant levels of ametryn degradation activity in crude lysates. A major protein band was observed by SDS-PAGE that migrated slightly faster than the intense bands of the chaperone proteins (data not shown). Ultimately, conditions were found to elute the His-tagged TrzN uniquely from a nickel column to yield a homogeneous protein. The enzyme was dialyzed to remove imidazole, which inhibited TrzN activity. Ten milligrams of pure protein was obtained from 1 liter culture. Yield was sacrificed to obtain purity.

The purified TrzN protein appeared homogeneous by SDS-PAGE, with an estimated subunit molecular weight of 53,000. Based on translation of the gene sequence, the native TrzN protein with the His tag was calculated to have a subunit molecular weight of 52,991. Gel filtration indicated a holoenzyme molecular weight of 50,000, suggesting that the native protein was a monomer. These data reveal that, if TrzN is a metalloenzyme, all the ligands to the metal(s) are provided by amino acids in a single polypeptide chain.

TABLE 1. Influence of zinc addition to growth medium on TrzN metal population and specific activity

Growth medium Zn(II) supplementation (μM)	Zn atom/TrzN subunit ratio[a]	TrzN sp act (μmol/min/mg)[b]
0	1:18	1.1
20	1:16	1.2
100	1:2	9.6
500	1:1	22.2

[a] Zinc concentration determined by inductively coupled plasma emission spectroscopy.
[b] Determined using ametryn as substrate.

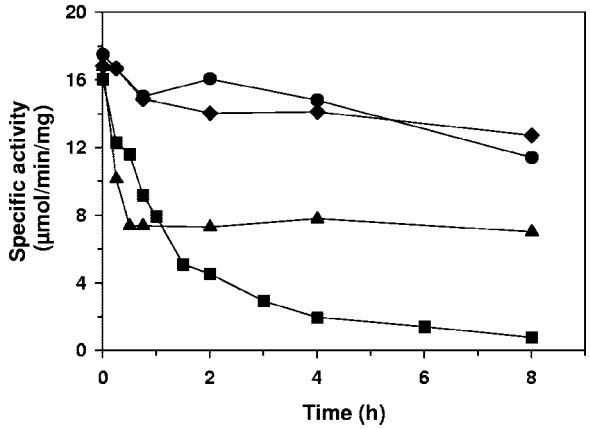

FIG. 3. Time-dependent loss of TrzN ametryn-hydrolysis activity following incubation with 5 mM metal chelators or a buffer control. Symbols: ◆, EDTA; ▲, 1,10-phenanthroline; ■, 8-hydroxyquinoline-5-sulfonic acid; ●, buffer control.

Metal determination experiments with native enzyme. In initial experiments, substoichiometric metal concentrations were detected, with zinc being observed in the greatest abundance. Addition of $ZnCl_2$ or $CoCl_2$ resulted in substantial increases in activity. The largest activity increases were observed with zinc, which, at saturation, typically yielded a specific activity of around 20 μmol/min per mg protein. However, different batches of purified, native TrzN showed highly variable specific activity, ranging from 1 to 20 μmol/min per mg protein.

In an attempt to overcome this variability, batches of recombinant *E. coli* cells were grown with increasing amounts of $ZnSO_4$ in the medium and TrzN was purified from each of the harvested cell pastes. The purified enzymes were then compared with respect to specific activity and zinc content (Table 1). A dramatic and asymptotic increase in specific activity of TrzN was observed with increasing zinc concentration in the growth medium. The highest specific activity of TrzN, obtained from cells grown with 500 μM zinc, was 22.2 μmol/min per mg protein, which is comparable to the maximum activity observed in the previously described zinc supplementation experiments. Metal determinations showed that the increase in specific activity was matched by a corresponding increase in the concentration of zinc in TrzN. The maximum activity was observed with a stoichiometry of one atom of zinc per enzyme subunit molecule.

Metal chelators remove metal and activity concomitantly. Native TrzN with high specific activity was treated with chelators or incubated without chelator under identical conditions as a control. Minimal diminution in activity was observed in the control or EDTA treatments. However, treatment with 1,10-phenanthroline or 8-hydroxyquinoline-5-sulfonic acid led to significant losses in enzyme activity over an 8-h period (Fig. 3). In subsequent experiments, TrzN was incubated with 8-hydroxyquinoline-5-sulfonic acid for 6 h, and activity and metal content were determined. After treatment, activity was approximately 4% of the original activity, and the zinc stoichiometry was less than 0.07 Zn per enzyme subunit. This preparation is referred to as "metal-depleted TrzN" in subsequent experiments.

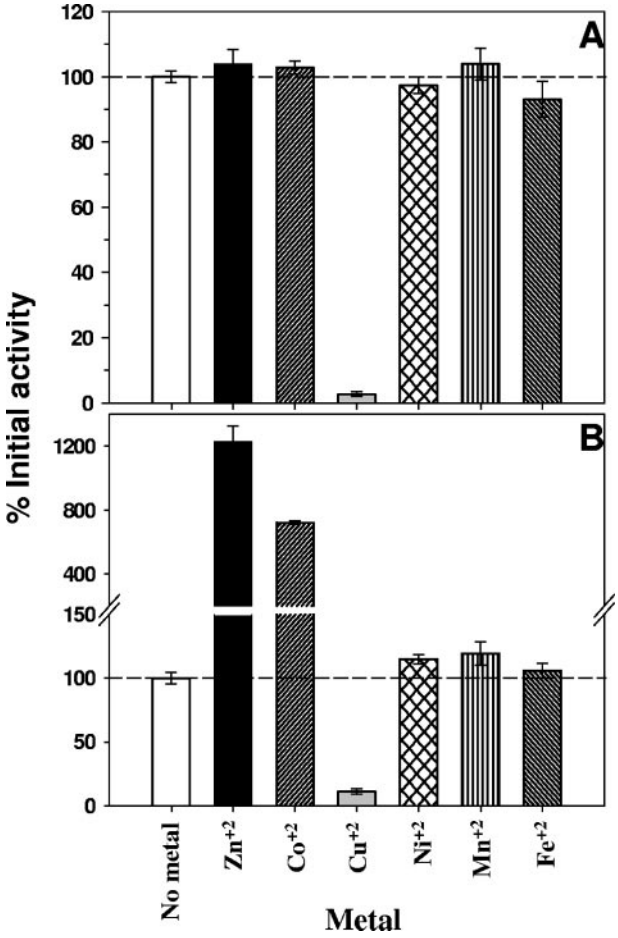

FIG. 4. Effect of metal addition on ametryn hydrolysis activity of native TrzN purified from recombinant cells grown on 500 μM zinc (A) and metal-depleted TrzN (B). All metals were added at a final concentration of 0.2 mM. Error bars represent the standard errors of the means.

Metal addition to native and metal-depleted TrzN. The effect of zinc, cobalt, copper, nickel, manganese, and iron addition on enzyme activity was tested with native (Fig. 4A) and metal-depleted (Fig. 4B) TrzN. Very little effect on the native enzyme was seen with any metal, except for copper, which showed marked inhibition (Fig. 4A). Metal addition to metal-depleted TrzN showed three differential effects (Fig. 4B). Marked inhibition was observed with copper, even to levels well below that of uninhibited metal-depleted TrzN. Cobalt and zinc strongly stimulated activity, with zinc showing the greatest stimulation. In contrast, iron, manganese, and nickel showed little or no effect. The activity of the zinc-amended metal-depleted enzyme was within 90% of the activity of the original enzyme prior to metal depletion.

Effect of metal on activity with different substrates. Native zinc-containing TrzN and cobalt-substituted TrzN were tested with several known substrates and one previously untested substrate (dipropetryn) (Fig. 5). Initially, metal-depleted enzymes were incubated with metals and tested for activity with different substrates over a period of time. As shown in the inset to Fig. 5, activity stabilized after about 30 min. Subsequent

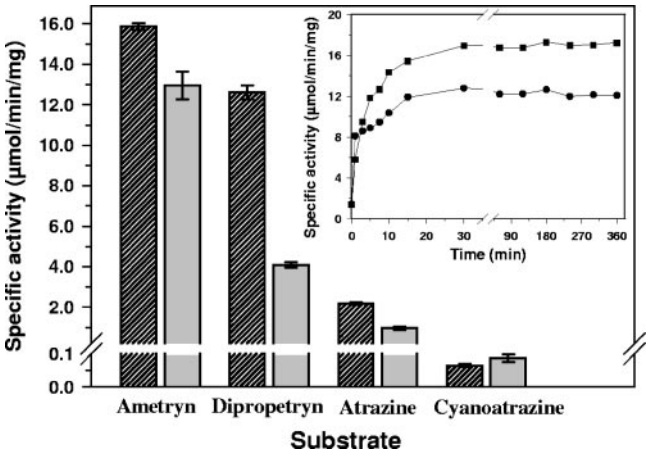

FIG. 5. Specific activity of Zn(II)- (▨) and Co(II)-reconstituted TrzN (▩) with selected *s*-triazines as substrates. Inset: specific activity of metal-depleted TrzN with ametryn following incubation with cobalt or zinc for up to 6 h; solid circles indicate Co-TrzN, and solid squares represent Zn-TrzN. Error bars represent the standard errors of the means.

preincubations with metals were carried out for 2 h prior to assay with substrates. With ametryn and atrazine, cobalt-substituted enzyme contained 80% and 45%, respectively, of the activity of the native enzyme with zinc, which was set at 100%. With dipropetryn, the activity of the cobalt-substituted TrzN was only 30% of that of the native enzyme. With cyanoatrazine, activity was modestly higher with the cobalt-substituted enzyme than with the zinc-substituted enzyme.

Steady-state kinetic parameters. In light of the experiments above, steady-state kinetic parameter assays were conducted with native enzyme that had one equivalent of zinc per subunit and negligible quantities of other metals. The substrates tested were major herbicides with which the activity is highest with zinc-containing TrzN (Table 2). The three lowest-activity substrates underwent chloride displacement. The k_{cat} and k_{cat}/K_m increased in the chlorinated *s*-triazine series: terbutylazine < simazine < atrazine. The higher-activity substrates contained an S-alkyl group rather than a chlorine atom. In general, the activities were approximately 1 order of magnitude higher for S-alkyl than for chloride displacement. The highest k_{cat}/K_m was observed with simetryn.

Thioatrazine (SH in place of the Cl atom of atrazine) was observed not to be a substrate for TrzN, but it did act as an inhibitor in admixture with atrazine or ametryn. Thioatrazine significantly inhibited TrzN activity; with both thioatrazine and

TABLE 2. Steady-state kinetic parameters for TrzN on selected *s*-triazine compounds

Compound	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Simetryn	3.4 ± 0.5	5.5 ± 0.03	1,618 ± 180
Ametryn	17.6 ± 1.9	13.3 ± 0.60	756 ± 67
Dipropetryn	45.6 ± 5.8	7.0 ± 0.51	154 ± 10
Atrazine	19.7 ± 0.4	2.1 ± 0.02	107 ± 1.2
Simazine	26.5 ± 1.0	1.8 ± 0.05	68 ± 0.9
Terbutylazine	17.8 ± 0.6	0.4 ± 0.01	22 ± 0.4

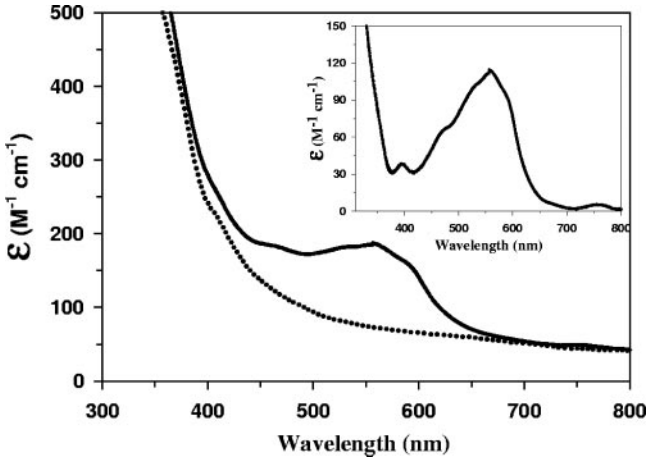

FIG. 6. UV-visible spectra of Co(II)-reconstituted (solid line) and metal-depleted (dotted line) TrzN. Inset: difference spectrum of Co(II)-reconstituted TrzN minus the spectrum of the metal-depleted enzyme.

ametryn present at 0.015 mM, activity was inhibited by 60%. Since the solubility is very limited (0.03 mM), extensive steady-state assays at several concentrations of thioatrazine as an inhibitor could not be conducted. However, using saturating thioatrazine concentrations and varying the ametryn concentration with and without inhibitor, two lines that intersected on the *y* axis were obtained on a double-reciprocal plot, suggesting that thioatrazine is a competitive inhibitor.

Electronic spectroscopy of cobalt-substituted TrzN. The electronic absorbance spectrum of 400 μM cobalt-substituted TrzN, prepared as described in Materials and Methods section, was obtained in buffered solution at pH 7.0 (Fig. 6). A comparison with metal-depleted enzyme allowed the calculation of a difference spectrum and an estimation of the extinction coefficient for the main absorption band of the cobalt center (Fig. 6, inset). The major band showed an absorption maximum at 550 nm with shoulders at 470, 520, and 600 nm. The extinction coefficient of the main band at 550 nm was 110 M^{-1} cm^{-1}.

Additional incubations were conducted with thioatrazine. The addition of thioatrazine increased the overall absorbance by 20%. Incubations of thioatrazine with cobalt salts alone or with metal-depleted enzyme did not give significant changes in absorbance.

DISCUSSION

Two enzymes have been identified that hydrolyze atrazine to hydroxyatrazine, AtzA and TrzN. TrzN has a much broader specificity than AtzA, showing activity in displacing cyano, azido, halide, S-alkyl, and O-alkyl substituents (20). The latter three substituents are found in major *s*-triazine herbicide classes, making TrzN an important enzyme for determining the environmental fate of these chemicals. Despite this importance, TrzN had been less well studied than AtzA. AtzA had been overexpressed in completely soluble form (2) and was shown to require one iron atom per AtzA subunit (16), and DNA shuffling had revealed critical residues in AtzA that influence its substrate specificity (9). No comparable studies had been conducted with TrzN until the present work.

While TrzN has been demonstrated to be present in many bacteria (7, 10, 21, 22), it had not been studied as extensively as AtzA. The original purification of TrzN from its native host yielded only 80 μg of protein (22). Subsequent recombinant expression efforts yielded no (5) or low (12, 20) activity. The latter efforts gave purified recombinant protein showing a specific activity with ametryn of 1.5 μmol/min per mg (20), compared to the reported activity of 18 μmol/min per mg protein with TrzN purified from the wild-type host (22).

The issue of whether TrzN was a metalloenzyme was rendered difficult to address due to limitations in obtaining sufficient quantities of enzyme for metal determinations and its variable activity. A variable-activity enzyme may show a low metal content due to poor insertion of metal into the binding site due to misfolding during overexpression in a recombinant host. This was resolved in the present study by using a double chaperone system for the heterologous expression of recombinant TrzN. In our previous studies (20), most of the recombinant TrzN was localized to insoluble inclusion bodies and had low activity, and soluble enzyme thus obtained could not be reconstituted by adding metal ions (20). In the current study, we now show that TrzN can be expressed at a high level in a soluble, properly folded form, with stoichiometric metal content and maximally observed enzyme activity.

Previous observations which suggested that TrzN was not a metalloenzyme can now be reconciled with other data in light of the studies conducted here. Previously, TrzN was expressed at a low level in its native *Nocardioides* host to yield an enzyme preparation that was likely fully populated with metal based on the specific activity that was reported (22). Similar activity levels were obtained here with purified TrzN from recombinant cells grown on 500 μM zinc that contained 1 mol of zinc per mole of subunit. This fully metal-populated TrzN was not activated by metal and was not affected by EDTA, similar to the findings of Topp et al. (22).

The present study unequivocally establishes TrzN as a metalloenzyme member of the amidohydrolase superfamily. The well-studied members of the superfamily typically contain one or two catalytically relevant metals (18). However, it cannot be assumed that all members of this superfamily will require a metal for activity. For example, the structure of uronate isomerase from *Thermotoga maritima* has been solved without a metal atom modeled into the structure (14), and metals have been shown only to inhibit activity (1). Moreover, the crystal structure of a TatD-related DNase from *Thermotoga maritima* has been solved without a bound metal (Protein Data Bank accession no. 1J6O). However, since the function of the TatD homolog has not been determined, its possible metal requirement cannot be determined currently.

In general, amidohydrolase superfamily members contain one or two metal atoms per subunit, with zinc being the most commonly identified metal. The enzyme functionally closest to TrzN is AtzA, and the native form of the latter enzyme contains iron. The closest amidohydrolase to TrzN, for which a structure is known, is *E. coli* cytosine deaminase, which also contains iron (8). Zinc is the physiologically relevant metal in AtzC (19). Now, TrzN was shown in the present study to use zinc physiologically. This was established in several ways. First, purified TrzN from cells grown in LB medium contained more zinc than any other metals. Second, when cells were grown in

medium with a high concentration of zinc, the enzyme had maximally observed enzyme activity, comparable to the activity reported by Topp et al. (22) using TrzN purified in small yields from wild-type cells. Third, quantitative determination of the metal content in highly active TrzN preparations revealed 1 mole of zinc per mole subunit and an insignificant level of any other metal. Fourth, metal-depleted enzyme was incubated individually with nickel, copper, iron, manganese, cobalt, and zinc. Only cobalt and zinc gave active enzyme, and zinc gave full activity while cobalt provided only about 50% of the original activity. Cobalt activates other amidohydrolase superfamily enzymes but is usually not the physiologically relevant metal. Cobalt is found at very low intracellular levels compared to zinc, and cobalt enzymes are rare (23).

The other outstanding issue is whether TrzN contains a binuclear or mononuclear metal center. The latter is suggested by the observation that the highest-activity enzyme that we obtained contained one metal per subunit and the enzyme is a monomer in solution. AtzA, AtzC, and cytosine deaminase are also proposed to be mononuclear metalloenzymes based on subunit stoichiometry. This conclusion for cytosine deaminase is also supported by X-ray structure determination (4). Adenosine deaminase is in the same domain of the amidohydrolase superfamily as TrzN, and this has also been established by X-ray crystallography to be a mononuclear zinc metalloenzyme (24). Elucidation of the structure of TrzN will more definitively resolve the issue of the metal center stoichiometry.

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ADDENDUM IN PROOF

After the manuscript was prepared, it came to our attention that the amidohydrolase superfamily member uronate isomerase does indeed function catalytically without a bound metal ion (L. Williams, T. Nguyen, Y. Li, T. N. Porter, and F. M. Raushel, Biochemistry **45:**7453–7462, 2006).

REFERENCES

1. **Ashwell, G., and A. J. Wahba.** 1960. Uronic acid metabolism in bacteria. I. Purification and properties of uronic acid isomerase in *Escherichia coli*. J. Biol. Chem. **235:**1559–1565.
2. **de Souza, M. L., M. J. Sadowsky, and L. P. Wackett.** 1996. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP: gene sequence, enzyme purification, and protein characterization. J. Bacteriol. **178:**4894–4900.
3. **Holm, L., and C. Sander.** 1997. An evolutionary treasure: unification of a broad set of amidohydrolases related to urease. Proteins **28:**72–82.
4. **Ireton, G. C., G. McDermott, M. E. Black, and B. L. Stoddard.** 2002. The structure of *E. coli* cytosine deaminase. J. Mol. Biol. **315:**687–697.
5. **Mulbry, W. W., H. Zhu, S. M. Nour, and E. Topp.** 2002. The triazine hydrolase gene *trzN* from *Nocardioides* sp. strain C190: cloning and construction of gene-specific primers. FEMS Microbiol. Lett. **206:**75–79.
6. **Pérez-Pérez, J., and J. Gutiérrez.** 1995. An arabinose-inducible expression vector, pAR3, compatible with ColE1-derived plasmids. Gene **158:**141–142.
7. **Piutti, S., E. Semon, D. Landry, A. Hartmann, S. Dousset, E. Lichtfouse, E. Topp, G. Soulas, and F. Martin-Laurent.** 2003. Isolation and characterisation of *Nocardioides* sp. SP12, an atrazine-degrading bacterial strain possessing the gene *trzN* from bulk- and maize rhizosphere soil. FEMS Microbiol. Lett. **221:**111–117.
8. **Porter, D. J. T., and E. A. Austin.** 1993. Cytosine deaminase: the role of divalent metals in catalysis. J. Biol. Chem. **268:**24005–24011.
9. **Raillard, S., A. Krebber, Y. Chen, J. E. Ness, E. Bermudez, R. Trinidad, R. Fullem, C. Davis, M. Welch, J. Seffernick, L. P. Wackett, W. P. Stemmer, and J. Minshull.** 2001. Novel enzyme activities and functional plasticity revealed by recombining highly homologous enzymes. Chem. Biol. **8:**891–898.
10. **Rousseaux, S., A. Hartmann, and G. Soulas.** 2001. Isolation and characterisation of new Gram-negative and Gram-positive atrazine degrading bacteria from different French soils. FEMS Microbiol. Ecol. **36:**211–222.
11. **Sadowsky, M. J., Z. Tong, M. L. de Souza, and L. P. Wackett.** 1998. AtzC is a new member of the amidohydrolase protein superfamily and is homologous to other atrazine-metabolizing enzymes. J. Bacteriol. **180:**152–158.
12. **Sajjaphan, K., N. Shapir, L. P. Wackett, M. Palmer, B. Blackmon, J. Tomkins, and M. J. Sadowsky.** 2004. *Arthrobacter aurescens* TC1 atrazine catabolism genes *trzN*, *atzB*, and *atzC* are linked on a 160-kilobase region and are functional in *Escherichia coli*. Appl. Environ. Microbiol. **70:**4402–4407.
13. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
14. **Schwarzenbacher, R., J. M. Canaves, L. S. Brinen, X. Dai, A. M. Deacon, M. A. Elsliger, S. Eshaghi, R. Floyd, A. Godzik, C. Grittini, S. K. Grzechnik, C. Guda, L. Jaroszewski, C. Karlak, H. E. Klock, Eric Koesema, J. S. Kovarik, A. Kreusch, P. Kuhn, S. A. Lesley, D. McMullan, T. M. McPhillips, M. A. Miller, M. D. Miller, A. Morse, K. Moy, J. Ouyang, A. Robb, K. Rodrigues, T. L. Selby, G. Spraggon, R. C. Stevens, H. van den Bedem, J. Velasquez, J. Vincent, X. Wang, B. West, G. Wolf, K. O. Hodgson, J. Wooley, and I. A. Wilson.** 2003. Crystal structure of uronate isomerase (TM0064) from *Thermotoga maritima* at 2.85 Å resolution. Proteins Struct. Funct. Genet. **53:**142–145.
15. **Seffernick, J. L., G. Johnson, M. J. Sadowsky, and L. P. Wackett.** 2000. Substrate specificity of atrazine chlorohydrolase and atrazine-catabolizing bacteria. Appl. Environ. Microbiol. **66:**4247–4252.
16. **Seffernick, J. L., H. McTavish, J. P. Osborne, M. L. de Souza, M. J. Sadowsky, and L. P. Wackett.** 2002. Atrazine chlorohydrolase from *Pseudomonas* sp. strain ADP is a metalloenzyme. Biochemistry **41:**14430–14437.
17. **Segel, I. H.** 1975. Other methods of plotting enzyme kinetics data, p. 208–224. *In* I. H. Segal (ed.), Enzyme kinetics: behavior and analysis of rapid equilibrium and steady-state enzyme systems. Wiley-Interscience, New York, N.Y.
18. **Seibert, C. M., and F. M. Raushel.** 2005. Structural and catalytic diversity within the amidohydrolase superfamily. Biochemistry **44:**6383–6391.
19. **Shapir, N., J. P. Osborne, G. Johnson, M. J. Sadowsky, and L. P. Wackett.** 2002. Purification, substrate range, and metal center of AtzC: the *N*-isopropylammelide aminohydrolase involved in bacterial atrazine metabolism. J. Bacteriol. **184:**5376–5384.
20. **Shapir, N., C. Rosendahl, G. Johnson, M. Andreina, M. J. Sadowsky, and L. P. Wackett.** 2005. Substrate specificity and colorimetric assay for recombinant TrzN derived from *Arthrobacter aurescens* TC1. Appl. Environ. Microbiol. **71:**2214–2220.
21. **Strong, L. C., C. Rosendahl, G. Johnson, M. J. Sadowsky, and L. P. Wackett.** 2002. *Arthrobacter aurescens* TC1 metabolizes diverse *s*-triazine ring compounds. Appl. Environ. Microbiol. **68:**5973–5980.
22. **Topp, E., W. M. Mulbry, H. Zhu, S. M. Nour, and D. Cuppels.** 2000. Characterization of *s*-triazine herbicide metabolism by a *Norcardioides* sp. isolated from agricultural soils. Appl. Environ. Microbiol. **66:**3134–3141.
23. **Wackett, L. P., W. H. Orme-Johnson, and C. T. Walsh.** 1989. Transition metal enzymes in bacterial metabolism, p. 165–206. *In* T. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, New York, N.Y.
24. **Wilson, D. K., F. B. Rudolph, and F. A. Quicho.** 1991. Atomic structure of adenosine deaminase complexed with a transition state analog: understanding catalysis and immunodeficiency mutations. Science **252:**1278–1284.