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Purification and Characterization of an Inducible *s*-Triazine Hydrolase from *Rhodococcus corallinus* NRRL B-15444R

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The widespread use and relative persistence of *s*-triazine compounds such as atrazine and simazine have led to increasing concern about environmental contamination by these compounds. Few microbial isolates capable of transforming substituted *s*-triazines have been identified. *Rhodococcus corallinus* NRRL B-15444 has previously been shown to possess a hydrolase activity that is responsible for the dechlorination of the triazine compounds deethylsimazine (6-chloro-*N*-ethyl-1,3,5-triazine-2,4-diamine) (CEAT) and deethylatrazine (6-chloro-*N*-isopropyl-1,3,5-triazine-2,4-diamine) (CIAT). The enzyme responsible for this activity was purified and shown to be composed of four identical subunits of 54,000 Da. Kinetic experiments revealed that the purified enzyme is also capable of deaminating the structurally related *s*-triazine compounds melamine (2,4,6-triamino-1,3,5-triazine) (AAAT) and CAAT (2-chloro-4,6-diamino-1,3,5-triazine), as well as the pyrimidine compounds 2,4,6-triaminopyrimidine (AAAP) and 4-chloro-2,6-diaminopyrimidine (CAAP). The triazine herbicides atrazine and simazine inhibit the hydrolytic activities of the enzyme but are not substrates. Induction experiments demonstrate that triazine hydrolytic activity is inducible and that this activity rises approximately 20-fold during induction.

The *s*-triazine herbicides have been used in a variety of weed control programs with major crops. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine [CIET]) is the leading member of this group and is used primarily for control of broad-leaved and grassy weeds in corn, sorghum, rangeland, sugarcane, pineapple, and turf grass sod. About 36×10^6 kg of atrazine is applied yearly in the United States (11). The intensive use of atrazine in some areas and its relative persistence have led to instances in which its levels in groundwaters and surface waters have exceeded Environmental Protection Agency maximum contaminant levels (1).

Few microorganisms have been isolated that metabolize alkylated *s*-triazines such as atrazine at rates that are suitable for environmental remediation (for reviews, see references 2, 5, 10, 14, and 16). Cook and Hutter (6) isolated and characterized an isolate of *Rhodococcus corallinus* that dechlorinates and deaminates the alkylated *s*-triazine deethylsimazine (6-chloro-*N*-ethyl-1,3,5-triazine-2,4-diamine [CEAT]) but not CIET (Fig. 1). They described the partial purification of two hydrolytic dechlorination activities from *Rhodococcus* extracts with native molecular sizes of 180,000 and 450,000 Da (7).

Our laboratory has been involved in the characterization and manipulation of bacterial activities for detoxifying pesticide wastes and remediating contaminated soils and water. The unique enzymatic activities displayed by *R. corallinus* may prove useful for specific bioremediation strategies. Although the herbicide atrazine is not a substrate for the *Rhodococcus* enzymes, manipulation of the genes responsible for degradation may yield enzymatic activities that do attack atrazine. In addition, gene probes for the *Rhodococcus* enzymes may prove useful for isolating related *s*-triazine degradation genes from other microbial isolates. In this report, we describe the purification and characterization of the *Rhodococcus* triazine hydrolase enzyme which is responsible for the dechlorination of CEAT and deamination of melamine (2,4,6-triamino-1,3,5-triazine [AAAT]).

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MATERIALS AND METHODS

Bacterial strain. *R. corallinus* NRRL B-15444R (5, 6) was obtained from the USDA National Center for Agricultural Utilization Research, Peoria, Ill., with permission of the depositors. For triazine hydrolase isolation, *R. corallinus* was grown at 30°C in minimal media containing 60 mM glycerol and 5 mM cyanuric acid as the carbon and nitrogen sources, respectively (7).

Chemical reagents. We have adopted the nomenclature of Cook (5) for the abbreviations of *s*-triazine compounds based on the substituents on the ring (Fig. 1). CIET, CEAT, deethylatrazine (6-chloro-*N*-isopropyl-1,3,5-triazine-2,4-diamine [CIAT]), simazine (CEET), *N*-ethylammeline (OEAT), and 2,4-dichloro-6-amino-1,3,5-triazine (CCAT) were graciously provided by CIBA-GEIGY, Greensboro, N.C. Stable stock solutions of CEAT (1 M), CIAT (1 M), CIET (0.5 M), CEET (0.1 M), CCAT (0.1 M), 2-chloro-4,6-diamino-1,3,5-triazine (CAAT [Aldrich]) (0.5 M), and 4-chloro-2,6-diaminopyrimidine (CAAP; [Aldrich]) (1 M) were prepared in dimethyl sulfoxide. A stock solution of 30 mM OEAT was prepared in 0.1 M HCl. Other triazine and pyrimidine substrates were obtained from Aldrich and were prepared as follows: ammeline (2,4-diamino-6-hydroxy-1,3,5-triazine [AAOT]), 10 mM in 0.1 N HCl; cyanuric acid (1,3,5-triazine-2,4,6-triol [OOOT]), 30 mM in 0.1 N NaOH; melamine, 10 mM in water; and 2,4,6-triaminopyrimidine (AAAP), 20 mM in water.

Purification of the triazine hydrolase. Cells from 20 liters of *Rhodococcus* culture which had been grown to stationary phase were pelleted by centrifugation ($4,000 \times g$ for 10 min at 4°C). The 20-g pellet (consisting of cells and a considerable amount of polysaccharide) was resuspended with 40 ml of ice-cold 10 mM potassium phosphate (pH 7.0) containing 0.1 mM $MgSO_4$

FIG. 1. Proposed pathway for the microbial degradation of the *s*-triazines (symmetrical triazines) atrazine, simazine, and melamine (adapted from reference 5). We have adopted the nomenclature of Cook (5) for the abbreviations of these compounds based on the substituents on the ring: A, amino; C, chloro; E, ethylamino; I, isopropylamino; O, hydroxy; T, triazine ring structure. *R. corallinus* is not capable of metabolizing atrazine or simazine but can metabolize deethylsimazine to *N*-ethylammelide. The *R. corallinus* triazine hydrolase described in this study is capable of dechlorinating CEAT and of deaminating AAAT.

(buffer A). This cell suspension was passed three times through a chilled French pressure cell (15,000 lb/in^2), and whole cells and debris were removed by centrifugation (12,000 $\times$ *g* for 10 min at 4°C). The supernatant was subjected to ultracentrifugation (105,000 $\times$ *g* for 1 h at 4°C), and the supernatant from this treatment (crude soluble fraction) was removed and used as a source of triazine hydrolase for further purification. The crude soluble fraction was pumped (3 ml/min) onto an Accell Plus quaternary methylamine (QMA) anion-exchange column (2.0 by 15 cm) (Waters/Millipore, Milford, Mass.) that had been equilibrated with buffer A. The column was washed with buffer A until all unbound material was eluted and a stable baseline was obtained. A linear gradient of 0 to 0.5 M potassium phosphate (pH 7.0) was then run (5 ml/min) to elute bound material from the column. Fractions containing triazine hydrolase activity were pooled and used for further purification.

The pooled QMA fractions containing triazine hydrolase activity were brought to 1 M $(NH_4)_2SO_4$ by the addition of solid $(NH_4)_2SO_4$ and pumped at 3 ml/min onto a TSK-phenyl column (2.15 by 15 cm; HP-Genenchem, South San Francisco, Calif.) that had been equilibrated with buffer A containing 1 M $(NH_4)_2SO_4$. The column was washed with equilibration buffer until all unbound material was eluted and a stable baseline was obtained. A linear gradient of 1.0 to 0 M $(NH_4)_2SO_4$ in buffer A was then run at 5 ml/min to elute bound material from the column. Peak active fractions were pooled and dialyzed against buffer A.

Size exclusion chromatography of phenyl fractions was carried out with a Protein-Pak 300SW column (7.8 by 300 mm; Waters) equilibrated and run with buffer A at 1 ml/min. Pooled phenyl fractions were concentrated by using Centricon-30 microconcentrators (Amicon, Beverly, Mass.), and 0.1 ml of this concentrate was injected onto the column. Fractions containing hydrolase activity were pooled. For estimation of the native molecular size of the hydrolase, the column was calibrated with freshly prepared protein standards (Combithek Calibration Proteins II; Boehringer Mannheim, Indianapolis, Ind.) run in buffer A.

Protein determinations and SDS-PAGE. Protein concentrations in crude extracts were determined by the method of Bradford (4). Protein concentrations in partially purified and purified enzyme preparations were determined by the spectrophotometric method of Kalb and Bernlohr (12). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the method of Laemmli (13).

Enzyme assays. Dechlorination of CEAT and CIAT was measured by monitoring the appearance of chloride (3), and then CEAT and CIAT were measured spectrophotometrically at 260 nm and 238 nm, respectively. The experimentally determined values for extinction coefficients for these substrates and their dechlorinated products were as follows: CEAT, ε_{260} = 5.2 mM^{-1} cm^{-1}; OEAT, ε_{260} < 0.1 mM^{-1} cm^{-1}; CIAT, ε_{238} = 6.0 mM^{-1} cm^{-1}; OIAT, ε_{238} = 12.5 mM^{-1} cm^{-1}. Deamination of AAAT, CAAT, CAAP, and AAAP were measured by monitoring the appearance of ammonia (18) and spectrophotometrically at characteristic wavelengths. The experimentally determined values for extinction coefficients for these substrates and their deaminated products were as follows: AAAT, ε_{235} = 2.5 mM^{-1} cm^{-1}; OAAT, ε_{235} = 6.9 mM^{-1} cm^{-1}; CAAT, ε_{243} = 2.6 mM^{-1} cm^{-1}; COAT, ε_{243} = 5.6 mM^{-1} cm^{-1}; AAAP, ε_{270} = 12.8 mM^{-1} cm^{-1}; OAAP, ε_{270} = 14.7 mM^{-1} cm^{-1}; CAAP, ε_{301} = 1.4 mM^{-1} cm^{-1}; COAP, ε_{301} = 12.9 mM^{-1} cm^{-1}. In all cases, reaction rates derived from spectrophotometric assays were in agreement with rates derived from chloride or ammonia release measurements. Typical assays contained 0.5 ml of buffer A with 0.25 mM CEAT or 0.5 mM AAAT and 2 to 50 μl of cell extract and were incubated at 37°C. To estimate inhibition constant (K_i) values for CIET and CEET, dechlorination rates of CEAT were determined by using reactions performed in the presence and absence of 50 μM CEET or 50 μM CIET, respectively. Lineweaver-Burk plots were then used to estimate K_i values.

TABLE 1. Purification of the *s*-triazine hydrolase from *R. corallinus*

Purification step	Total protein (mg)	Total activity[a] (μmol/min)	Sp act (μmol/min/mg of protein)	Recovery (%)	Purification (fold)
Crude soluble	155.4	22.6	0.146	100	
QMA	21.7	20.1	0.925	89	6.3
TSK-phenyl	1.85	9.62	5.20	42	35
300SW	0.030	0.218	7.28	—[b]	49

[a] Triazine hydrolase activity was determined by using assays with CEAT. Hydrolase assays with AAAT gave comparable results, but the total AAAT deamination activity at each step was approximately 40-fold higher than the CEAT dechlorination activity (data not shown).

[b] Only a small portion of the TSK-phenyl-purified material was subjected to 300SW chromatography. Of the portion which was run, the recovery of hydrolase activity was 66%.

Induction experiments. Cultures (1 liter) of *R. corallinus* that had been grown to stationary phase in Luria broth (LB) at 30°C were harvested by centrifugation, washed twice in 0.5 volume of buffer A, resuspended in 0.25 volume of minimal medium (6) containing 60 mM glycerol, and aliquoted into flasks containing one or more of the following components: LB (20 g/liter), ammonium sulfate (3 mM), urea (3 mM), biuret (3 mM), OOOT (2 mM), AAAT (1 mM), CAAT (0.5 mM with 0.1% dimethylsulfoxide), CEAT (0.5 mM with 0.05% dimethyl sulfoxide), dimethyl sulfoxide (0.1%), glutamate (6 mM), and glutamine (3 mM). Resuspended cells were incubated for 24 h at 30°C with shaking and then harvested by centrifugation. Harvested cells were washed twice with buffer A, resuspended with buffer A, and disrupted by sonication (three pulses of 400 W for 15 s each at 4°C). Unbroken cells and cell debris were removed by centrifugation as described above. Triazine hydrolase assays were performed by using the resultant crude supernatant and CEAT as the hydrolase substrate.

RESULTS

Enzyme purification. The triazine hydrolase was purified by chromatography of the crude soluble fraction on a QMA anion-exchange column, followed by chromatography of active fractions on TSK-phenyl and 300SW columns (Table 1). The final preparation had a specific activity approximately 50 times that of the starting material and yielded a single band of approximately 54,000 Da when subjected to SDS-PAGE (Fig. 2).

Enzyme characterization. The native molecular size of the triazine hydrolase was estimated by size exclusion chromatography to be approximately 200,000 Da. These results suggest that the enzyme is a tetramer composed of four identical subunits of 54,000 Da.

As noted by Cook and Hutter (7), the *Rhodococcus* triazine hydrolase required no cofactors (although 100 μM $MgSO_4$ was typically included in most buffers). In assays designed to test the effect of different metal salts or divalent metal chelators, $CaSO_4$, $MnSO_4$, $MgSO_4$, $MoSO_4$ or EDTA showed no effect on enzyme activity at 100 μM. In contrast, assays with 100 μM $CoSO_4$ or $ZnSO_4$ showed approximately 50% inhibition of hydrolase activity. Moreover, $CuSO_4$ was a potent inhibitor of the triazine hydrolase, with about 50% inhibition of activity at 5 μM and 100% inhibition at 20 μM (data not shown). To estimate the substrate specificity of the triazine hydrolase, Michaelis-Menten constants were estimated from least-squares regression of Woolf plots by using structurally related *s*-triazines and pyrimidines (Fig. 3). Although the enzyme showed no detectable dechlorination activity toward CIET or CEET, kinetic experiments revealed that these compounds competitively inhibited the dechlorination of CEAT. Lineweaver-Burk plots were used to estimate K_i values for CEET and CIET by using CEAT as the substrate (Fig. 3). Taking the K_m values (for CIAT and CEAT) and K_i values (for CIET and CEET) as relative measures of the affinity of the enzyme toward these compounds, there is a significant difference between the affinities for the monoalkylated triazines CEAT and CIAT and the dialkylated triazines CEET and CIET. Moreover, the enzyme is able to dechlorinate only the monoalkylated triazines CEAT and CIAT. It displayed no detectable dechlorination activity in assays containing the triazine CEET, CIET, CCAT, or CAAT or the pyrimidine CAAP.

Kinetic experiments revealed that the purified *Rhodococcus* triazine hydrolase is also capable of deaminating the structurally related *s*-triazine compounds melamine and CAAT, as well as the pyrimidine compounds AAAP and CAAP. Among these substrates, the enzyme displays a pronounced preference for *s*-triazine over pyrimidine compounds. In addition, there is a preference for nonchlorinated (AAAP and AAAT) over chlorinated (CAAP and CAAT) compounds. In each case, analysis of the reaction products revealed that only one amino group had been hydrolyzed from the substrates (data not shown). The enzyme displayed no detectable deamination activity in assays with the triazine OEAT, OAAT, CCAT, CIAT, or CEAT.

Hydrolase induction. Cook and Hutter previously noted that one of the *Rhodococcus* hydrolase activities was inducible, with optimal activity coming from cells grown with AAAT or OOOT as the nitrogen source (7). We examined the induction

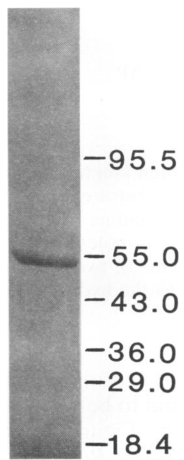

FIG. 2. Determination of the subunit molecular mass of the triazine hydrolase from *R. corallinus*. The purified hydrolase was subjected to SDS-PAGE as described in the text. Molecular masses of protein standards are expressed in kilodaltons.

COMPOUND	K_m (μM)	V_{max} (μmole/min-mg protein)	K_I (μM)
DECHLORINATION REACTIONS			
CEAT	82	2.4	----
CIAT	61	0.76	----
CIET	n.d.	0	385
CEET	n.d.	0	746
DEAMINATION REACTIONS			
AAAT	840	270	-----
CAAT	2100	23	-----
AAAP	33	0.36	-----
CAAP	240	0.12	-----

FIG. 3. Michaelis-Menten kinetic constants (K_m, V_{max}, K_i) for the triazine hydrolase from *R. corallinus* with triazine and pyrimidine substrates. Abbreviations for triazine substrates are explained in the legend to Fig. 1. Abbreviations for pyrimidine substrates follow the same nomenclature: A, amino; C, chloro; P, pyrimidine ring structure. n.d., not determined. A zero in the V_{max} column means that no activity could be detected. The limit of detection was <0.01 μmole/min/mg of protein. Deamination of AAAP could occur at any one of three different positions, and deamination of CAAP could occur at one of two positions. The specific sites of deamination have not been determined. K_i values were determined by using assays containing CEAT as the hydrolase substrate as described in the text.

of *Rhodococcus* triazine hydrolase activity further to quantify the level of induction and to better understand the basis of the induction phenomenon. Figure 4 shows the induction of triazine hydrolase activity in *Rhodococcus* cells that had been grown on rich medium (LB broth) and then were washed and incubated for 24 h in minimal medium containing various nitrogen sources. Hydrolase activity was highest in cells incubated in the presence of CAAT, followed by cells incubated in the presence of AAAT and OOOT. Incubation with biuret (the product of triazine ring cleavage), urea, glutamine, glutamate, or ammonium sulfate leads to much lower levels of hydrolase activity. Results with cells incubated with AAAP, CAAP, or no added nitrogen source were identical to those with cells incubated with ammonium sulfate (data not shown). Incubation with CEAT did not lead to higher levels of hydrolase induction than did incubation of control cells incubated with

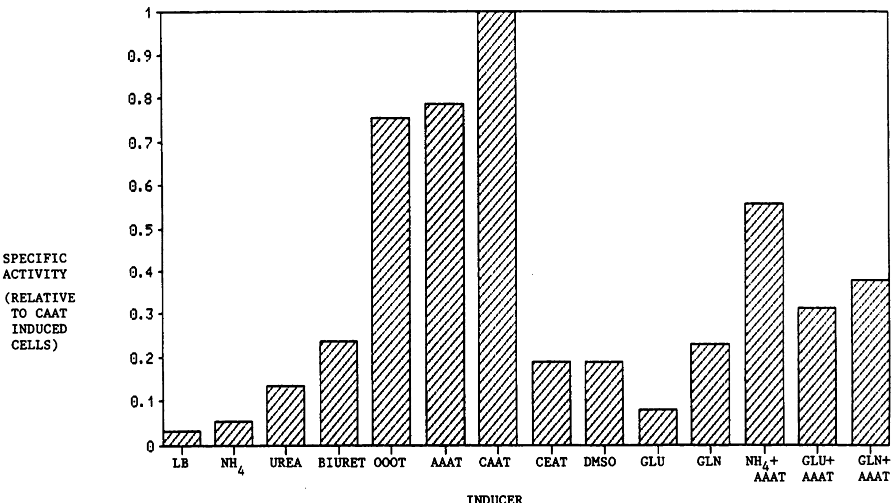

FIG. 4. Induction of triazine hydrolase activity after incubation of *Rhodococcus* cells in medium containing different nitrogen sources. Cells were grown in LB medium as described in the text, washed, resuspended, and incubated in medium containing one or more compounds that could serve as a source of nitrogen. Triazine hydrolase activity was determined by using assays with CEAT and cell extracts prepared from the induced cells. Hydrolase assays with AAAT and the cell extracts gave identical results (data not shown). The specific activities shown have been normalized relative to those of extracts induced by CAAT. The compounds used as sources of nitrogen during induction are indicated below the bars. LB, Luria broth; NH_4, ammonium sulfate; DMSO, dimethyl sulfoxide (control for dimethyl sulfoxide contained in CAAT and CEAT solutions); GLU, glutamate; GLN, glutamine.

dimethyl sulfoxide (the solvent used to dissolve CEAT). Results with CIAT, CIET, and CEET were identical to those with CEAT (data not shown). Incubation of cells with AAAT and ammonium sulfate led to hydrolase levels approximately 60% as high as with AAAT alone. Incubation of cells with AAAT and either glutamate or glutamine led to hydrolase levels approximately 40% as high as with AAAT alone.

DISCUSSION

This report describes the purification of a unique 200,000-Da *s*-triazine hydrolase from extracts of *R. corallinus* NRRL B-15444R that is capable of dechlorinating the triazine CEAT (and CIAT) and of deaminating the *s*-triazine AAAT (and CAAT). These results are somewhat at variance with those of Cook and Hutter (7), who isolated *R. corallinus* NRRL B-15444R. In the brief communication of results of the study that initially characterized the hydrolytic activities of *R. corallinus*, Cook and Hutter described two enzyme fractions (molecular sizes, 180,000 and 450,000 Da) from gel filtration chromatography of *Rhodococcus* extracts that dechlorinated CEAT. They also reported that there was an AAAT deamination activity which coeluted with the 450,000-Da dechlorinase on gel filtration and ion-exchange columns (7). Unlike the previous authors, we observed no evidence of multiple triazine dechlorinases, even when the *Rhodococcus* cells were cultured in medium containing AAAT rather than OOOT. Although we do not understand these differences in the results, three lines of evidence suggest that the enzyme that we have isolated from *Rhodococcus* extracts is responsible for both the CEAT dechlorination and AAAT deamination activities. First, both activities copurify through four distinct purification steps to yield a single polypeptide by SDS-PAGE. Moreover, protein sequencing of the N-terminal region of the purified protein demonstrated the presence of only one amino acid sequence (data not shown). Second, both activities are inhibited by the triazines CIET and CEET and by the metal salts $CoSO_4$, $ZnSO_4$, and $CuSO_4$. Finally, induction experiments showed identical patterns of induction for CEAT and AAAT hydrolase activities (data not shown).

Induction experiments with *Rhodococcus* cells grown on rich medium and then transferred into minimal medium with different nitrogen sources revealed that triazine hydrolase activity increases approximately 20-fold after incubation in the presence of CAAT, AAAT, or OOOT. Biuret, which is the ring cleavage product of OOOT, was a poor inducer of triazine hydrolase. However, induction apparently requires more specificity than just an intact *s*-triazine ring, since CEAT and related alkylated *s*-triazines were relatively poor inducers compared with CAAT. Incubation of *Rhodococcus* cultures in the presence of AAAT along with alternate nitrogen sources (ammonium sulfate, glutamine, or glutamate) demonstrated that these compounds repress hydrolase induction compared with incubation with AAAT alone. Thus, like other enzymatic activities associated with nitrogen metabolism, triazine hydrolase activity in *R. corallinus* is probably regulated at several levels.

The ability of the triazine hydrolase to dechlorinate substrates such as CEAT makes this enzyme unique and potentially useful for environmental remediation. However, our kinetic and induction data suggest that the primary activity of the triazine hydrolase is toward compounds that can be deaminated (such as AAAT and AAAP). If this is true, isolation of other microbial strains that degrade these compounds may reveal additional hydrolytic enzymes with similarly broad substrate ranges.

Although *Pseudomonas* genes specifying the degradation of nonalkylated *s*-triazines such as ammeline have been cloned and expressed in *Escherichia coli* (8, 9), the molecular basis of the degradation of alkylated triazines such as CEAT is not understood. Indeed, the scarcity of such microbial activities has slowed the basic understanding of these processes as well as severely limited the development of bioremediation strategies for treating triazine-contaminated sites. However, there are recent reports of bacterial isolates capable of degrading CIET

and other alkylated *s*-triazines (2, 14, 15, 17). Like the enzyme from *R. corallinus*, these activities are inducible depending on the nitrogen source. Characterization of these new activities and their comparison with the *Rhodococcus* hydrolase may lead to a better understanding of how such activities arise and how they may be manipulated for environmental remediation.

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