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Ring dechlorination of deethylsimazine by hydrolases from *Rhodococcus corallinus*

(*s*-Triazines; degradative pathway; isozymes)

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1. SUMMARY

Cell-free extracts of *Rhodococcus corallinus* NRRL B-15444R dechlorinated deethylsimazine (2-chloro-4-ethylamino-1,3,5-triazine-6-amine) to *N*-ethylammeline (2-amino-4-ethylamino-1,3,5-triazine-6(5H)-one) anaerobically, and, thus, presumably hydrolytically. Two enzyme fractions (M_r about 450 000 and 180 000) that dechlorinated deethylsimazine were detected after gel permeation chromatography of cell extracts.

2. INTRODUCTION

Specific ring dechlorinations at the aromatic nucleus are still rare, dechlorination subsequent to aerobic ring cleavage being better characterized [1]. Nevertheless, quantitative dehalogenation concomitant with dioxygenation is established [2] as is hydrolytic dechlorination [3,4] and, anaerobically, reductive ring dechlorination [e.g., 5].

Deethylsimazine (CEAT, Fig. 1) is a product of the aerobic degradation of the *s*-triazine herbicides simazine and atrazine [6–8]. Typical of non-hydroxylated derivatives of *s*-triazines, it has considerable aromatic character, but, in contrast to the benzenoid ring, delocalization of the π-electrons is not complete [9] and hydrolytic removal of substituents is known [10–12]. We have demonstrated biodegradation of CEAT, involving dechlorination and deamination to EOOT in the coryneform bacterium *R. corallinus* [13], and we now confirm that the initial reaction is a quantitative, hydrolytic, ring dechlorination by two isofunctional, but different hydrolases.

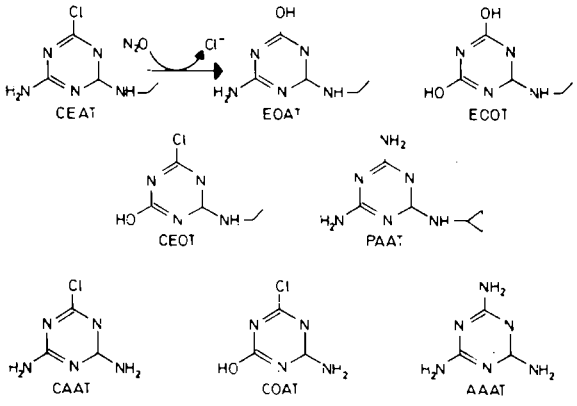

Fig. 1. The dechlorination reaction and *s*-triazines mentioned in the text. *s*-Triazines are abbreviated in the system proposed by Cook [11]: A, amino; C, chloro; E, ethylamino; I, isopropylamino; O, hydroxy; P, cyclopropylamino; T, *s*-triazine ring.

3. MATERIALS AND METHODS

3.1. Materials and analyses

The *s*-triazines (obtained from Ciba-Geigy) and the abbreviations used are shown in Fig. 1. The compounds were of high purity [13,14] and were determined by reversed phase high pressure liquid chromatography (HPLC) [14,15]. Chloride ion was determined colorimetrically [16].

3.2. Organism, cell suspensions and enzyme assays

The organism used was *R. corallinus* NRRL B-15444R [13]. Due to a shortage of CEAT, only precultures (60 ml) were grown in CEAT-glycerol-mineral salts medium [13]. A whole preculture was used to inoculate a 0.9-l culture in a simple fermenter [17], with either 5 mM OOOT and 30 mM glycerol or 5 mM AAAT and 60 mM glycerol in salts medium; cells were harvested in the late exponential phase. Cells were washed twice in salts medium and stored frozen. Cell suspensions, cell-free extracts, discontinuous enzyme assays with 0.5 mM substrate, and anaerobic work in a glovebox (< 2 ppm O_2) were prepared and carried out essentially as described previously [10,11]. CEAT is somewhat acid-labile, spontaneously dechlorinating to EOAT on prolonged storage in 0.1 M HCl [13]. However, CEAT was stable in the short-term exposure to acid when enzyme reactions were stopped with perchloric acid (and samples stored at pH 7), or when Cl^- was measured colorimetrically. CAAT, CEOT and COAT were subject to immediate spontaneous dechlorination on exposure to perchloric acid or the chloride assay: these compounds could be assayed by directly injecting portions from enzyme assays onto HPLC columns.

3.3. Column chromatography

Crude extract was centrifuged (110000 $\times$ *g*) for 2 h at 4°C and 15 ml (about 150 mg of protein) applied to a Sephadex G-200 column (93 $\times$ 2.6 cm at 4°C). Protein was eluted in 10 mM potassium phosphate buffer pH 7.2 containing 0.25 mM $MgSO_4$ at 18 ml/h, and 4.1-ml fractions were collected. Centrifuged crude extract (15 ml) was also applied to a Sephacryl S-300 column (90 $\times$ 2.6 cm at 4°C), eluted with the phosphate buffer at 62 ml/h and collected in 5-ml portions. The column was calibrated with M_r standards (Boehringer) under identical conditions. Portions from the higher-M_r fractions were pooled (20 ml) and applied on a 30 $\times$ 2.6 cm DEAE-Sepharose column at 4°C. The starting buffer was the elution buffer from the gel filtration, and proteins were eluted at 60 ml/h (5-ml fractions) with a linear gradient (800 ml) to 500 mM potassium phosphate.

4. RESULTS AND DISCUSSION

Cell extracts from *R. corallinus* catalysed the dechlorination of CEAT to EOAT, small quantities of which were deaminated to EOOT (Fig. 2). The substrate was stable in the absence of enzyme and the enzyme preparation did not interfere with the determination of *s*-triazines. Cell extracts (and whole cells) did not dechlorinate CEOT, the putative alternative intermediate to EOOT. So we believe there to be only one degradative pathway from CEAT to EOOT, namely quantitative dechlorination followed by a deamination of the type described by Cook et al. [11]. The specific activity of the dechlorination in the cell extracts was 0.9 mkat/kg of protein, somewhat higher than the 0.3 mkat/kg of protein calculated for growing cells [13], so we presume we are indeed

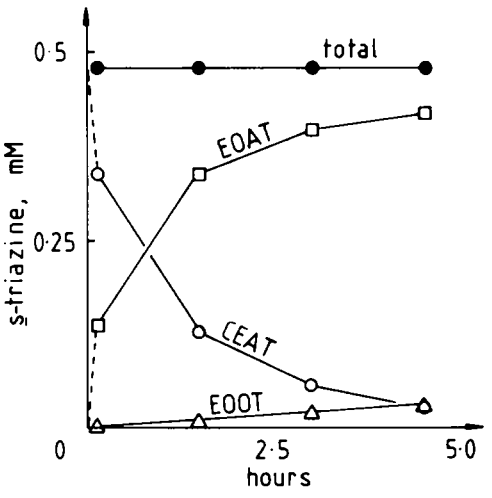

Fig. 2. Stoichiometry of conversion of CEAT to EOAT and EOOT by crude extracts of *R. corallinus*.

studying the enzyme(s) responsible for dechlorination in whole cells.

The dechlorination was catalysed by soluble enzymes, because the activity was present only in the supernatant fluid after ultracentrifugation (110000 × *g* for 2 h). No cofactors were required, because the removal of small molecules by chromatography on Sephadex G-25 columns left significant activity in the preparation (0.4 mkat/kg of protein). The dechlorination was catalysed both anaerobically and aerobically by whole cells and by extracts. We therefore presume the dechlorination to be a hydrolytic substitution at the aromatic ring, analogous to other quantitative reactions at the *s*-triazine ring [10–12] and chemically feasible because of the low electron density at the ring carbon atoms [9].

Chromatography of the 110000 × *g* supernatant fluid on Sephadex G-200 showed 2 fractions which were eluted close to the void volume, and both of which dechlorinated CEAT (Fig. 3) to EOAT (identified by HPLC and UV spectra; cf. [13]) and chloride ion; no EOOT was observed. CIAT was also a substrate for both fractions (not shown) yielding IOAT. The non-alkylated homologue, CAAT, was not dechlorinated by either fraction; negligible amounts of OAAT were formed. CAAT, however, was deaminated by the fraction of M_r 450000 (Fig. 2): the product was COAT (identified by HPLC and UV spectra; cf. [12]).

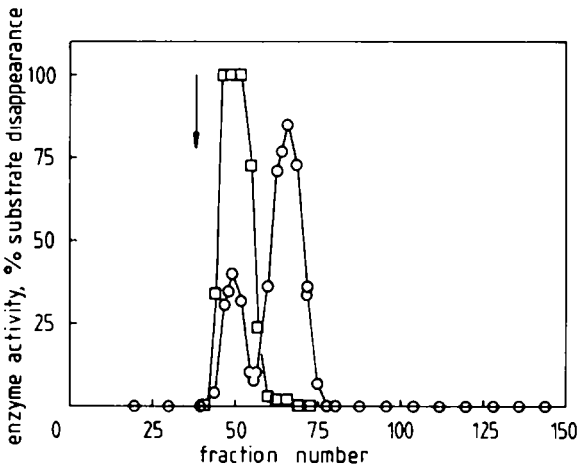

Fig. 3. Chromatography on Sephadex G-200 of CEAT-dechlorination in soluble proteins from a crude extract of OOOT-grown cells of *Rhodococcus corallinus*. The arrow indicates the void volume. Dechlorination of CEAT (○); deamination of CAAT (□).

Chromatography on Sephacryl S-300 (not shown) gave M_r values of about 450000 and 180000 for the 2 fractions, both of which could dechlorinate CEAT, but not CAAT. The larger protein fraction could deaminate AAAT and CAAT as well as dechlorinate CEAT, and these 3 activities co-chromatographed on DEAE-Sepharose, where the protein(s) eluted with about 0.2 M buffer. The relative amounts of activity of the 2 protein fractions varied with the method of culture; AAAT-grown cells gave much more of the larger protein and OOOT-grown cells more of the smaller protein.

Dechlorination of CEAT in extracts of *R. corallinus* seems to be catalysed by two different hydrolases. We presume there to be one enzyme (M_r approx. 180000) which is specific for CEAT (and CIAT) but does not dechlorinate the non-alkylated analogue CAAT. We postulate a second enzyme (M_r approx. 450000) which also dechlorinates CEAT and CIAT but not CAAT. In addition, there is an enzyme which co-elutes with the high-M_r dechlorinase on gel permeation and ion exchange columns, and which deaminates CAAT and AAAT analogous to the AAAT-amidohydrolase from *Pseudomonas* spp. NRRL B-12227 (M_r 200000), an enzyme which deaminates AAAT, PAAT and CAAT [12]. It is as yet unclear how many enzymes of approx. M_r 450000 are involved.

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