Bacterial Organomercurial Lyase: Overproduction, Isolation, and Characterization†

Tadhg P. Begley, Alan E. Walts, and Christopher T. Walsh*
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
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ABSTRACT: Organomercurial lyase mediates the first of two steps in the microbial detoxification of organomercurial salts. This enzyme encoded on the plasmid R831 obtained from *Escherichia coli* J53-1 has been overproduced to the level of 3% of the soluble cell protein in *E. coli* by a construction using the T7 promoter. The enzyme has been purified to homogeneity in quantity in three steps. It is a monomer of M_r 22 400 with no detectable cofactors or metal ions. It catalyzes the protonolysis of the C–Hg bond in a wide range of organomercurial salts (primary, secondary, tertiary, alkyl, vinyl, allyl, and aryl) to the hydrocarbon and mercuric ion with turnover rates in the range of 1–240 min^{-1}.

Organomercurials are toxic to most living systems (McAuliffe, 1977). They occur in nature as chemical pollutants. The neurotoxic methylmercuric salts arise from the biological methylation of mercury in sewage and sediments (Ridley et al., 1977). Bacteria have evolved a variety of strategies to detoxify heavy metals (Williams & Silver, 1984; Summers, 1985; Foster, 1983; Brown, 1985). Some metals cannot enter the cell (e.g., Ag^+; Silver, 1981), others are actively transported out of the cell (e.g., Cd^{2+}, arsenate; Perry & Silver, 1982; Mobley & Rosen, 1982; Silver & Keach, 1982), while still others are sequestered by tight-binding ligands within the cell (e.g., Cu^{2+}–metallothionein; Kagi & Nordberg, 1979). The microbial organomercurial resistance system utilizes two unusual enzymes to effect detoxification. The first, organomercurial lyase, catalyzes the protonolysis of the carbon–mercury bond to give hydrocarbon and mercuric ion. The second enzyme, mercuric ion reductase, catalyzes the reduction of Hg^{2+} by NADPH to zerovalent mercury, which evaporates from the culture (Figure 1). Resistance to inorganic Hg^{2+} ions but not organomercurials is provided by this second enzyme.

The inorganic mercury resistance systems ("narrow spectrum") of *Pseudomonas* (encoded on the transposon TN501) and of *Shigella* (encoded on the plasmid R100) have been extensively studied (Robinson & Tuovinen, 1984). The mer operon of both systems has been sequenced (Barrineau et al., 1985; Brown et al., 1983; Misra et al., 1985). Induction of the system with subtoxic levels of Hg^{2+} leads to the synthesis of four proteins in the R100 system (merT, P, C, and A) and to the synthesis of at least three proteins in the TN501 system (merT, P, and A). Both the merT and merP gene products are required for mercury transport into the cell. Mercuric ion reductase is the merA gene product. This enzyme is a flavoprotein and has been extensively studied in this laboratory (Fox & Walsh, 1982, 1983; Schultz et al., 1985). The functions of merC and merD are unknown.

The "broad-spectrum" organomercurial resistance system in *Escherichia coli* has not been as thoroughly studied as the narrow-spectrum Hg^{2+} resistance system. This system has been found on a large plasmid (R831, 90kb), and the mercuric ion resistance and the organomercurial resistance (merB) loci have been mapped by transposon mutagenesis (Ogawa et al., 1981). The mercuric ion reductase has been isolated, and it is similar to the TN501 enzyme (Schottel, 1978). The partial purification of organomercurial lyase encoded by R831 has been previously reported (Schottel, 1978). However, we have repeatedly found that this enzyme in low concentrations was unstable during chromatography. An organomercurial lyase from *Pseudomonas* K62 has also been described (Tezuka & Tonomura, 1976, 1978), but we find that this strain no longer produces the C–Hg bond cleaving enzyme, perhaps due to loss of a plasmid.

Mechanistic studies on organomercurial lyase sit at the interface of enzymology and organometallic chemistry. Carbon–metal bonds are rare in biological systems, where the best studied example is the carbon–cobalt bond of vitamin B_{12} (Halpern, 1985). Carbon–nickel bonds may be utilized by the enzyme carbon monoxide dehydrogenase in carbonyl insertion processes (Ragsdale & Wood, 1985). Organomercurial salts, unlike many other carbon–metal bond containing compounds, are quite stable to protonolysis. We find that less than 1% protonolysis of methylmercuric chloride occurs after treatment with concentrated hydrochloric acid for 100 min. The task of the enzyme is therefore not a trivial one. While the mechanism of nonenzymic protonolysis of dialkylmercurials has been thoroughly studied and found to occur by an S_E2 mechanism (Jensen & Rickborn, 1968), relatively little is known about the mechanism of the much slower protonolysis of organomercurial salts. Carbanion, radical, and carbocation species have all been proposed as intermediates with various substrates (Jensen & Rickborn, 1968).

In this paper we report the construction of an overproducing strain for the R831 organomercurial lyase and the isolation and characterization of substantial quantities of the pure enzyme, while in the following paper we detail mechanistic studies in support of an S_E2 pathway for the enzymatic protonolysis (Begley et al., 1986).

Experimental Procedures

Materials

Restriction enzymes T4 DNA ligase and T4 DNA polymerase were obtained from New England Biolabs. Calf intestinal alkaline phosphatase was from Boehringer Mannheim Biochemicals. Mercuric reductase was purified as described by Fox and Walsh (1982). *E. coli* JM101 was obtained from New England Biolabs. *E. coli* JM101(pGP1-2) and the plasmid pT7-4 was a gift from S. Tabor (Tabor & Richardson, 1985). Plasmid pCT4 carrying the gene for organomercurial

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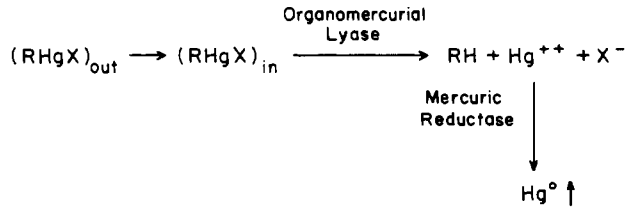

FIGURE 1: Organomercurial detoxification pathway.

lyase from *E. coli* R831 was a gift from Anne Summers (Ogawa et al., 1984). Buffer 1 consisted of 50 mM sodium phosphate, 0.5 mM EDTA, and 0.02% sodium azide (pH 6.9). Buffer 2 consisted of 10 mM bis-tris-propane,[1] and 0.1 mM EDTA (pH 7.5). Buffer 3 consisted of 0.1 M sodium phosphate and 10 mM L-cysteine (pH 7.4). This buffer is unstable due to cysteine oxidation and was freshly prepared before use. Buffer 4 consisted of 50 mM bis-tris-propane, 50 mM CAPS, and 10 mM 2-mercaptoethanol titrated to the required pH. The protease inhibitors were those described by Santi (D. Santi, University of California, San Francisco, personal communication). Solution A consisted of 200 mg of phenanthroline, 200 mg of benzamidine, and 1 mg of PMSF in 1 mL of ethanol. Solution B consisted of 1.2 mg of soybean trypsin inhibitor, 1.2 mg of aprotinin, and 0.5 mg of leupeptin in 0.25 mL of water. The protein solution was made 1% in A and 1% in B. Antibiotics were obtained from Sigma Chemical Co. Methylmercuric chloride was from Alfa; phenylmercuric acetate was from Aldrich; $^{203}HgCl_2$ was from New England Nuclear. The isomeric butylmercurials and the vinylmercurial were prepared by quenching the corresponding Grignard reagent as described in Makarova and Nesmeyanov (1967). Solutions of the organomercurials in Me_2SO (0.1 M) were used for enzymology. These solutions were stored at 0 °C. The gas chromatograph was a Carle Hach Model AGC100. The cells were grown in a New Brunswick shaker.

Methods

Construction of Plasmid pT7-4B. Plasmid pCT4 was digested with *Apa*I, blunt ended by treatment with T4 DNA polymerase in the presence of dGTP, and finally digested with *Sal*I followed by *Pst*I. Digestion with *Pst*I eliminated the necessity for gel purification of the merB-containing fragment. Plasmid pT7-4 was digested first with *Sal*I and then with *Sma*I and finally dephosphorylated with calf intestinal alkaline phosphatase. The *Sal*I/blunt *Apa*I fragment from pCT4 and the large *Sal*I/*Sma*I fragment from pT7-4 were ligated, and recombinant plasmid transformants of *E. coli* JM101 were selected on LB Amp plates (50 μg of Amp/mL; Maniatis et al., 1982). Plasmids from several viable colonies were isolated and characterized by restriction enzyme mapping with *Eco*RI and *Sal*I (2.3- and 2.5-kb fragments; Figure 2).

DNA after each reaction was purified by phenol extraction followed by ethanol precipitation (Maniatis et al., 1982). The restriction enzyme buffers and the plasmid minipreparation were from Maniatis et al. (1982). The transformation procedure was from the New England Biolabs M13 cloning manual.

Overproducing Strain. Plasmid pT7-4B was used to transform *E. coli* JM101(pGP1-2). Transformants were selected on Kan–Amp plates (LB medium, 50 μg/mL of each antibiotic) incubated at 30 °C. The plasmid composition of viable transformants was confirmed by agarose gel electrophoresis of the mixture of plasmids isolated by minipreparation (pT7-4B, 4.9 kb; pGP1-2, 7.4 kb).

Bacterial Growth Conditions. An overnight starter culture of *E. coli* JM101 (pT7-4B, pGP1-2) (LB medium, 30 °C) was diluted 150-fold into LB medium containing kanamycin (50 μg/mL) and ampicillin (50 μg/mL), grown at 30 °C in a New Brunswick shaker (200 rpm) to an optical density (590 nm) of 2.1. The culture was then heat shocked (42 °C, 1 h), incubated at 30 °C for a further 3 h, and harvested by centrifugation (6000*g*, 15 min). Ten liters of culture yielded 48 g of frozen cell paste.

Enzyme Assays. The enzyme was assayed by following methane production from CH_3HgCl by gas chromatography. The assay mixture consisted of 0.5 mL of buffer 3, 20 μL of mercuric reductase (1.2 mg/mL), 20 μL of NADPH (20 mM), 50 μL of organomercurial lyase solution (1.2 mg/mL), 10 μL of CH_3HgCl solution, and 400 μL of H_2O. All components were mixed, sealed in a 5-mL Wheaton vial, and incubated at 37 °C. Aliquots of the headspace (50 μL) were removed at various time intervals and analyzed for methane by gas chromatography [80% Porapak N/20% Porak Q (0.25 in. × 8 ft), 130 °C]. The instrument was calibrated with known quantities of methane. Assays for other gaseous products were similarly carried out. Benzene (from phenylmercuric acetate) was assayed by carrying out the reaction in the presence of 100 μL of dodecane and analyzing 2-μL aliquots of the organic phase by gas chromatography [4.1% Carbowax on Chromosorb G (0.25 in. × 10 ft), 120 °C, toluene used as an internal standard]. For the determination of kinetic parameters the volume of organomercurial added was varied. In each case a lag time of approximately 15 min was observed before the rate of hydrocarbon formation became linear. The enzyme loses approximately 50% activity after 60 min at 37 °C.

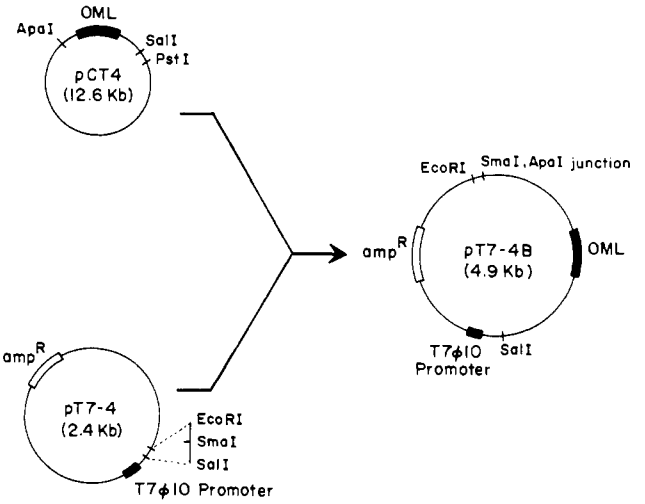

FIGURE 2: Construction of plasmid pT7-4B.

Table I

purification step	total act. (U)	total protein (mg)	sp act. (U/mg)[a]	yield (%)
crude extract	2400	1250	2	100
first DEAE-Sepharose	2420	65	37	100
second DEAE-Sepharose Ultrogel AcA44	1360	25	55	56
	1260	21	60	52

[a] 1 U = 1 nmol of CH_4/min.

[1] Abbreviations: Amp, ampicillin; Kan, kanamycin; PMSF, phenylmethanesulfonyl fluoride; kb, kilobase; dGTP, 2′-deoxyguanosine 5′-triphosphate; bis-tris-propane, 1,3-bis[[tris(hydroxymethyl)methyl]-amino]propane; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

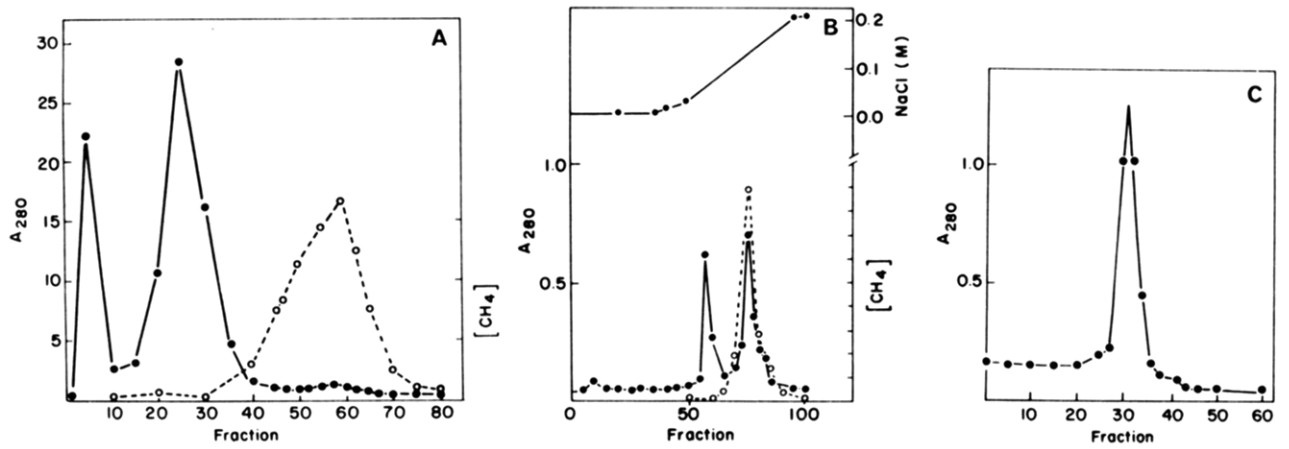

FIGURE 3: Purification of organomercurial lyase: (A) first DEAE-Sepharose chromatography; (B) second DEAE-Sepharose chromatography; (C) Ultragel AcA44 chromatography. The protein concentration (●) was measured by A_{280}. Methane formation (○) was measured as described under Methods.

Purification of Organomercurial Lyase. All columns were run at 4 °C. The results of the purification are summarized in Table I.

Step 1: Preparation of Cell-Free Extract. Frozen cells (10 g) were resuspended in 20 mL of buffer 1 and disrupted by passing twice through a french press at 12 000 psi. Protease inhibitors were added, and the suspension was spun at 27000*g* for 30 min.

Step 2: First DEAE-Sepharose Chromatography. The red supernatant from step 1 was applied to a DEAE-Sepharose CL6B column (2.5 × 37 cm^2) equilibrated in buffer 1. The column was eluted with the same buffer (1.7 mL/min, 7-mL fractions). The elution profile is shown in Figure 3A. Fractions containing organomercurial lyase were pooled (fractions 40–75) and concentrated to 20 mL (Amicon, PM-10 membrane).

Step 3: Second DEAE-Sepharose Chromatography. The enzyme from step 2 was dialyzed against buffer 2, applied to a DEAE-Sepharose CL6B column (2.5 × 11 cm^2), equilibrated in the same buffer, and eluted with a 0–0.2 M NaCl gradient (1.3 mL/min, 6.5-mL fractions). The elution profile is shown in Figure 3B. Fractions containing organomercurial lyase (68–80) were pooled and concentrated to 15 mL.

Step 4: Gel Filtration on Ultrogel AcA44. The enzyme from step 3 was applied to an Ultrogel AcA44 column (2.5 × 33 cm^2) equilibrated in buffer 1. The enzyme was eluted with the same buffer (0.8 mL/min, 4.0-mL fractions). The elution profile is shown in Figure 3C. Fractions 27–36 were pooled. The enzyme from step 4 was homogeneous by polyacrylamide gel electrophoresis (Figure 4).

pH–Rate Profile for Ethylmercuric Chloride. The reaction mixture for determining V_{max} and K_m at various pH values (7.0–10.9) consisted of 950 μL of buffer 4 and 50 μL of organomercurial lyase (0.56 mg/mL). The concentrations of ethylmercuric chloride used were between 2 and 0.5 mM. The reaction was followed by measuring ethane formation by GC. Each K_m and V_{max} value was determined in duplicate. The organomercurial is stable in the absence of enzyme in the pH range studied.

Attempted Alkylation with Iodoacetic Acid. Organomercurial lyase (50 μL, 0.56 mg/mL) was added to 450 μL of buffer 4 containing 1 mM 2-mercaptoethanol. After 1 h at room temperature, 10 μL of iodoacetic acid solution (10^{-1} M) was added. Enzymatic activity was measured after 6 h. A control sample without iodoacetic acid was also prepared.

Stoichiometry of Hg^{2+} Binding. Organomercurial lyase (300 μL of 0.9 mg/mL, 40 μM) was incubated at room temperature for 1 h with $^{203}HgCl_2$ (1.82 mM, 4.23 mCi/mmol), in the absence of thiol. The sample was then loaded onto an Ultrogel AcA44 column (30 cm × 1 cm) and eluted with phosphate buffer (50 mM, pH 7.4, 0.5-mL fractions). Aliquots (100 μL) were analyzed for protein by the Bradford assay and for ^{203}Hg by scintillation counting.

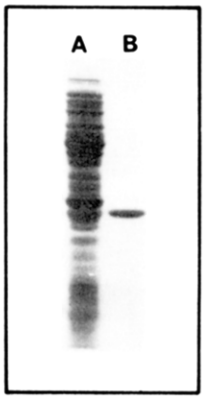

FIGURE 4: SDS–polyacrylamide gel of (A) total cell protein and (B) purified organomercurial lyase (25 μg). The total cell protein sample was prepared as described in Tabor and Richardson (1985). The gel was prepared as described under Methods.

Protein Determination. Protein concentration, unless otherwise stated, was determined by the method of Lowry et al. (1951) using bovine serum albumin and carbonic anhydrase as standards. The absorbance at 280 nm was used to monitor column fractions.

Polyacrylamide Gel Electrophoresis. Denaturing gels were run according to the Laemmli procedure (Laemmli, 1970). The separating gel and the stacking gel were 10% and 6% polyacrylamide, respectively. The gels were stained with Coomassie blue R.

Molecular Weight Determination. The subunit molecular weight was determined by SDS–polyacrylamide gel electrophoresis using the following standards: ovalbumin (43 000), α-chymotrypsinogen (25 700), β-lactoglobulin (18 400), and lysozyme (14 300). The molecular weight of the native enzyme was determined by gel filtration through a column of Ultrogel AcA44 (1.6 × 46 cm, 20 mL/h, 1 mg of enzyme) using the following standards: blue dextran (void volume), albumin (67 000), ovalbumin (43 000), chymotrypsinogen (25 000), and ribonuclease (13 700).

Amino Acid Composition and Amino-Terminal Analysis. These analyses were carried out by Genetic Design, Watertown, MA, and by Dr. John Wunderlich at the sequencing

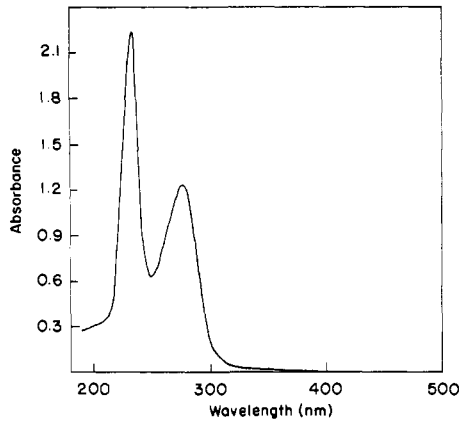

FIGURE 5: Electronic spectrum of organomercurial lyase. The protein concentration was 1.24 mg/mL.

facility at the University of Georgia, respectively.

Analysis for Metal. Neutron activation analysis of organomercurial lyase was performed by Dr. Ilhan Olmez at the MIT nuclear reactor. Qualitative data were obtained on pure enzyme (1 mg) that had been extensively dialyzed against doubly distilled deionized water and lyophilized. Separate counts with live time durations of 300, 900, and 20 000 s were done and failed to detect any significant quantities of metals above control background samples.

RESULTS

Construction of the Overproducing Strain. The overproduction strategy described herein involved inserting the T7 promoter in front of the cloned *E. coli* R831 derived organomercurial lyase gene (Tabor & Richardson, 1985). Cells containing this plasmid and a second plasmid containing the T7 RNA polymerase gene under P_L control constituted the overproducing strain. Heat shock derepressed the P_L promoter, leading to T7 RNA polymerase synthesis. This enzyme then enables efficient gene expression from the T7 promoter. This system has the advantage that T7 RNA polymerase does not recognize *E. coli* transcriptional termination signals so that a gene separated from the T7 promoter by an intervening sequence of undetermined length still has a good chance of being overproduced (Dunn & Studier, 1983). Plasmid pT7-4B was constructed by ligating the *Sal*I/blunt *Apa*I fragment of plasmid pCT4 with the large *Sal*I/*Sma*I fragment of plasmid pT7-4 (Figure 2). This recombinant plasmid was then used to transform *E. coli* JM101(pGP1-2) to give the overproducing strain. It turns out that 3% of the crude extract-soluble protein consisted of organomercurial lyase after induction as described under Methods.

Properties of Organomercurial Lyase. (*1*) *Molecular Weight.* The subunit molecular weight was found to be 22 400 by SDS–polyacrylamide gel electrophoresis. The apparent molecular weight by gel filtration of the native enzyme is 32 000. Thus, we conclude that the enzyme is a monomer of 22 400 Da.

(*2*) *Ultraviolet–Visible Spectrum.* The electronic spectrum of the enzyme is shown in Figure 5. The spectrum is that of a simple polypeptide. Organomercurial lyase does not require a chromophoric cofactor.

(*3*) *Metal Ion Analysis.* Qualitative neutron activation analysis indicated the absence of metal ions in the pure enzyme. We analyzed for all metallic elements except lead, magnesium, calcium, molybdenum, iridium, and tin, all of which are difficult to detect by this method. For the other elements the sensitivity was greater than 0.1 metal ion/enzyme.

Table II: Amino Acid Composition and N-Terminal Amino Acid Sequence

residue	residues/ molecule	residue	residues/ molecule	residue	residues/ molecule
Asx	14	Ala	29	Phe	8
Thr	17	Val	17	Lys	6
Ser	16	Met	5	His	6
Glx	23	Ile	9	Arg	16
Pro	15	Leu	28	Cys	4
Gly	15	Tyr	5		

N-Terminal Amino Acid Sequence

Met-Lys-Leu-Ala-Pro-Tyr-Ile-Leu-Glu-Leu-Leu-Thr-Ser-Val-Asn-Arg-Thr-Asn-Gly-Thr-Ala-Asp-Leu-Leu-Val-Pro-Leu-Leu-Arg-Glu-Leu-Ala-Lys-Gly-(-)-Pro-Val-Ser-(-)-(-)-Thr-Leu-Ala-Gly-Ile-Leu-Asp-(-)-Pro-Ala

Table III

substrate	V_{max} (nmol mg^{-1} min^{-1})	K_m (mM)	turnover no. (min^{-1})
methylmercuric chloride	31	0.5	0.7
n-butylmercuric chloride	880	0.9	20
sec-butylmercuric bromide	110	0.8	2.5
tert-butylmercuric chloride	40	0.8	0.9
phenylmercuric acetate	670	0.9	15.0
crotylmercuric bromide[a]	2600	1.3	58
vinylmercuric bromide	530	1.0	12
cis-2-butenylmercuric chloride[a]	10700	3.3	240
dimethylmercury	0		0

[a] See Begley et al. (1986) for details of these substrates.

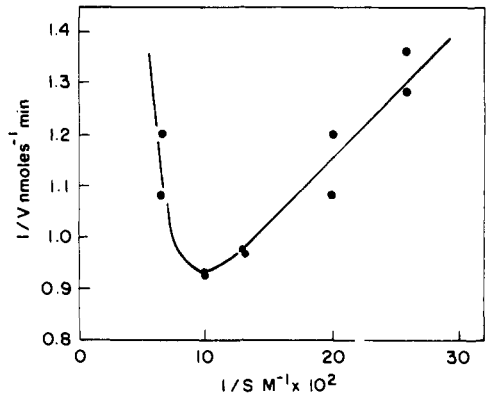

FIGURE 6: Lineweaver–Burk plot for the reaction of organomercurial lyase with methylmercuric chloride. The assay was carried out as described under Methods. The concentration of methylmercuric chloride used were 1.5, 1.0, 0.75, 0.5, and 0.38 mM.

(*4*) *Amino Acid Composition.* The amino acid composition and the amino-terminal sequence are summarized in Table II. These will be of value in setting the reading frame of the gene sequence. The 46 N-terminal residues identified account for about one-fifth of the total primary sequence. The DNA sequencing of this organomercurial lyase gene is in progress (A. Summers, personal communication).

(*5*) *Kinetic Parameters for Organomercurial Lyase.* The enzyme can be assayed for cleavage of C–Hg bonds by analysis of the hydrocarbons generated, in a discontinuous gas chromatographic assay. The lineweaver–Burk plot for organomercurial lyase activity with methylmercuric chloride is shown in Figure 6. The reaction showed strong substrate inhibition above 1 mM concentration of substrate for reasons as yet undetermined. The kinetic parameters are given in Table III.

(*6*) *Thiol Dependence.* The rate of the enzymatic protonolysis reaction is dependent on both the structure and the concentration of the buffer thiol. The rate for cysteine (5 mM) is 8 times the rate for 2-mercaptoethanol (5 mM), and no

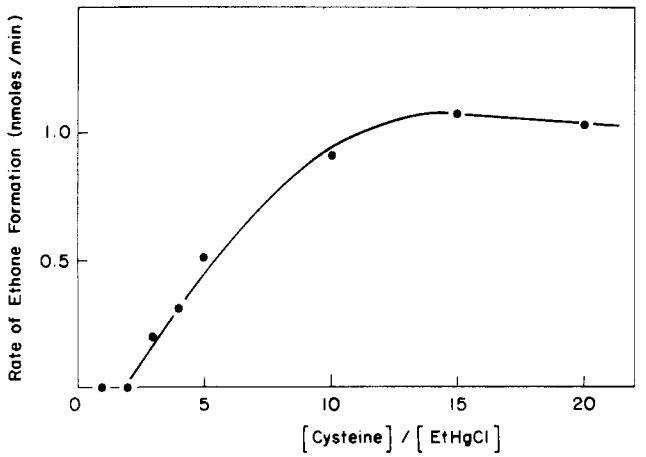

FIGURE 7: Dependence of the rate of ethylmercuric chloride (1 mM) protonolysis on the concentration of cysteine in the buffer. The assay was carried out as described under Methods.

protonolysis occurs with 2-aminoethanethiol. The effect of cysteine concentration on the rate is shown in Figure 7. At concentrations less than 2 mM no protonolysis occurs. At concentrations greater than 2 mM the rate increases with increasing cysteine concentration and levels out above 15 mM.

(*7*) *Substrate Specificity.* The kinetic parameters for several substrates are shown in Table III. The enzyme catalyzes the protonolysis of a wide range of organomercurials. We also observed substrate inhibition for *tert*-butylmercuric chloride above 1 mM but not for the other mercurials.

(*8*) *pH–Rate Studies.* The pH–rate profile for V_{max} and V_{max}/K_m was determined with ethylmercuric chloride as substrate. The rate of the reaction increased up to pH 10.2 when denaturation of the enzyme occurred. The apparent pK_a of the ascending arm of the profile was found to be 9.0 from a plot of log V_{max}/pH (Figure 8). The K_m for the reaction was found to be independent of pH.

(*9*) *Iodoacetic Acid Inactivation.* Given the fact that Hg^{2+} is one of the products and it has high affinity for thiols, we analyzed the susceptibility of the enzyme to inactivation by alkylating agents. In the event the enzyme was not inactivated by iodoacetic acid.

(*10*) *Stoichiometry of Hg^{2+} Binding.* We noted that at low buffer thiol/RHgX ratios the enzyme lost activity during turnover. It seemed likely that this could be due to accumulation of enzyme as Enz–Hg^{2+} at the end of turnover. To test this explicitly, we incubated the enzyme in thiol-free buffer with $^{203}Hg^{2+}$, followed by gel filtration. Indeed the inhibited enzyme issuing from the column contained 1 equiv of associated $^{203}Hg^{2+}$, suggesting a single binding site. Subsequent addition of thiol regenerates enzyme activity.

Discussion

The instability of the original organomercurial lyase producing *Pseudomonas* K62 strain (Tezuka & Tonomura, 1976, 1978) and the instability of the *E. coli* R831 enzyme during chromatography with small amounts of activity in crude extracts necessitated the construction of an overproducing strain before we could develop a reliable and reproducible purification scheme for obtaining pure, active, unproteolyzed enzyme. We chose the T7 promoter system as we did not know the position of the organomercurial lyase gene on the 2.5-kb fragment derived from plasmid pCT4 (Ogawa et al. 1984). The T7 RNA polymerase does not recognize *E. coli* termination signals (Dunn & Studier, 1983), so we expected that this system would give higher levels of overproduction than with other promoters (for example the tac promoter). In particular the distance of the tac promoter from the start codon is critical, and transcriptional termination before the organomercurial lyase gene is transcribed as a potential problem. In the event, the T7 overproducing strain synthesizes organomercurial lyase to the level of 3% of the soluble cell protein.

The enzyme was then readily purified (Table I). The greatest purification was achieved with the first DEAE-Sepharose column; the enzyme loses some activity on the second DEAE-Sepharose column. Attempts to substitute this column with a phenyl-Sepharose column or with a mono Q column were unsuccessful. In addition, the enzyme is unstable in crude extract, and addition of protease inhibitors is essential to isolate intact, active catalyst.

Organomercurial lyase has a subunit molecular weight of 22 400 as determined by SDS gels. The native molecular weight as determined by gel filtration was 32 000. We conclude that the enzyme is a monomer in its active form. The high value of the molecular weight observed in the gel filtration experiment may mean that the enzyme is a nonglobular protein. The molecular weight of the purified enzyme is considerably lower than that previously reported for the partially purified enzyme (43 000; Schottel, 1978) but corresponds to a 23 600-Da size predicted by a *Staphylococcus aureus* organomercurial lyase gene sequence (Silver, 1986). In *E. coli* minicells containing the plasmid R831 two polypeptides of 22 000 and 24 000 Da were produced (Jackson & Summers, 1982). This raises the possibility that the enzyme we have isolated is a processed polypeptide. This will be resolved when

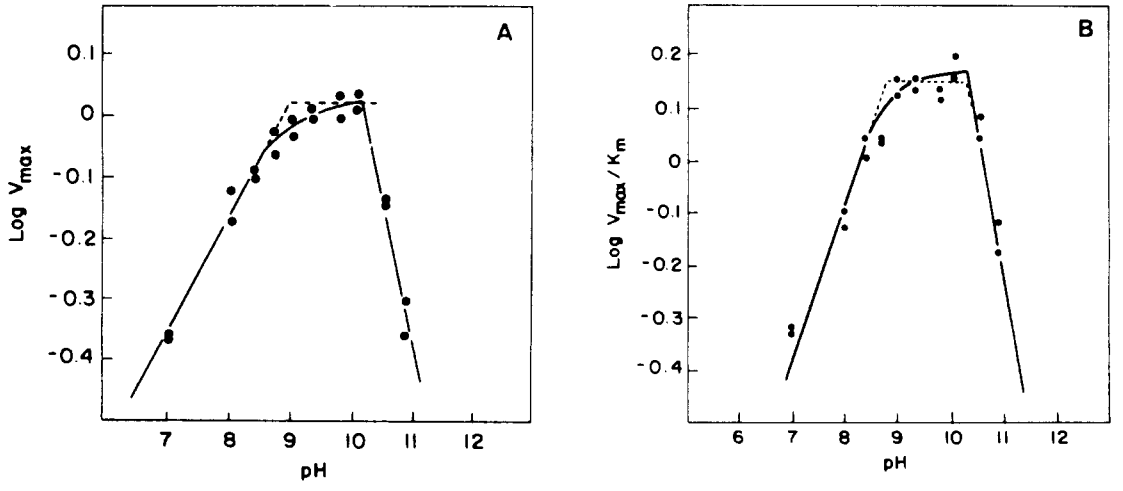

FIGURE 8: Plot of (A) log V_{max} vs. pH and (B) log V_{max}/K_m vs. pH for organomercurial lyase using ethylmercuric chloride as substrate.

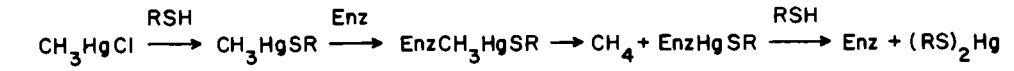

FIGURE 9: Mechanistic scheme for the organomercurial lyase catalyzed protonolysis of organomercurial salts.

the gene sequence is available.

The electronic spectrum of the enzyme is that of a simple polypeptide. No chromophoric cofactor was detected. This is consistent with the role of the enzyme as a simple protonolytic catalyst toward susceptible carbon–mercury bonds. Neutron activation analysis revealed the absence of bound metal ions. All metallic elements except lead, magnesium, calcium, molybdenum, iridium, and tin could have been detected.

The amino acid composition indicates the presence of four cysteines. It is not yet known whether these are involved in binding of the organomercurial substrate during catalysis or in chelation of product. Mercuration of the enzyme may cause some kinetic anomalies as exemplified in the initial rate lag and strong substrate inhibition as shown in the Lineweaver–Burk plot for methylmercuric chloride (Figure 6). Failure to inactivate the enzyme with iodoacetic acid suggests that any active site thiols if present are not unusually reactive. However, the enzyme does bind 1 equiv of $^{203}Hg^{2+}$ tightly in the absence of buffer thiol and is released only in the presence of a large excess of buffer thiol.

We had three possibilities for the enzyme assay: A coupled assay using mercuric reductase catalyzed reduction of Hg^{2+} by NADPH was found to give curved Lineweaver–Burk plots with arylmercurials. In addition, the rate of the uncoupled reaction of mercuric ion reductase with oxygen (2–4% of the rate of Hg^{2+} reduction; Fox & Walsh, 1982) was faster than the rate of alkylmercurial protonolysis because of the low turnover number of the lyase. Therefore, this assay was not of use in determining kinetic parameters. The ^{203}Hg volatilization assay (Schottel, 1978) in which the mercuric ion formed from the protonolysis reaction is reduced enzymatically or chemically with $SnCl_2$ to Hg^0, which then evaporates from the reaction mixture, is not sufficiently general for our purposes due to the technical difficulty of synthesizing radioactive organomercurials. We therefore resorted to a gas chromatographic assay of the hydrocarbon products which, although discontinuous, has the advantage of generality and great sensitivity (picomole range).

Organomercurial lyase catalyzed protonolysis of ethylmercuric chloride shows a surprisingly high optimum pH (10.2) for an enzyme that catalyzes a protonolysis reaction. The possible mechanistic significance of this will be discussed in the following paper (Begley et al., 1986). A plot of V_{max}/pH has a slope of only 0.2/pH and indicates the presence of a catalytically important residue of $pK_a = 9.0$. The unusually low slope suggests that the catalytic group can perform its function in both the ionized and the nonionized states or that more than one microscopic pK_a is involved. The enzymatic reaction is critically dependent on both the concentration and the structure of the buffer thiol. This is consistent with the minimal mechanism outlined in Figure 9. Rapid substitution (Rabinstein & Reid, 1984; Rodriguez et al., 1978; Eigen & Wilkins, 1965) of the counterion of the organomercurial salt by the buffer thiol gives an organomercurial thiolate complex that is probably the actual substrate for the enzyme. Hence, the dependence of the rate on thiol structure. We have found that the reaction works best with cysteine and does not work with 2-aminoethanethiol. After the cleavage of the C–Hg bond the buffer thiol facilitates demercuration of the enzyme–Hg^{2+} complex. It is not clear at this point why such a large excess of thiol over organomercurial (15/1) is required to give the optimum reaction rate or why 2 equiv of thiol is required to initiate the reaction.

Organomercurial lyase shows broad substrate specificity (Table II). Thus methyl-, primary, secondary, and tertiary alkyl-, aryl-, allyl-, and vinylmercurials are all substrates for this enzyme. On the other hand, dimethylmercury is not protonolyzed. The turnover numbers (1–240 min^{-1}) are on the low end of catalytic turnover rates, and the K_m values vary only slightly for the different substrates. The broad substrate specificity and the availability of large quantities of the pure enzyme have set the stage for challenging the enzyme with a range of mechanistically interesting substrates to elucidate the mechanism of this unique organometal-cleaving enzyme in physical organic terms, and this is the subject of the following paper (Begley et al., 1986).

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Registry No. MeHgCl, 115-09-3; BuHgCl, 543-63-5; *s*-BuHgBr, 868-82-6; *t*-BuHgCl, 38442-51-2; PhHgOAc, 62-38-4; MeCH=CHCH$_2$HgBr, 18355-68-5; CH$_2$=CHHgBr, 16188-37-7; *cis*-MeCH=CHCH$_2$HgCl, 104489-70-5; Me$_2$Hg, 593-74-8; organomercurial lyase, 72560-99-7.

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Mechanistic Studies of a Protonolytic Organomercurial Cleaving Enzyme: Bacterial Organomercurial Lyase†

Tadhg P. Begley, Alan E. Walts, and Christopher T. Walsh*
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received April 14, 1986; Revised Manuscript Received June 17, 1986

ABSTRACT: Mechanistic studies of the protonolytic carbon–mercury bond cleavage by organomercurial lyase from *Escherichia coli* (R831) suggest that the reaction proceeds via an S_E2 pathway. Studies with stereochemically defined substrates *cis*-2-butenyl-2-mercuric chloride (**1**) and *endo*-norbornyl-2-mercuric bromide (**2**) reveal that a high degree of configurational retention occurs during the bond cleavage, while studies with *exo*-3-acetoxynortricyclyl-5-mercuric bromide (**3**) and *cis-exo*-2-acetoxy-bicyclo[2.2.1]hept-5-enyl-3-mercuric bromide (**4**) show that the protonolysis proceeds without accompanying skeletal rearrangement. Kinetic data for the enzymatic reactions of *cis*-2-butenyl-2-mercuric chloride (**1**) and *trans*-1-propenyl-1-mercuric chloride (**6**) indicate that these substrates show enhanced reaction rates of ca. 10–200-fold over alkylvinylmercurials and unsubstituted vinylmercurials, suggesting that the olefinic methyl substituent may stabilize an intermediate bearing some positive charge. Enzymatic reaction of 2-butenyl-1-mercuric bromide (**5**) yields a 72/23/5 mixture of 1-butene/*trans*-2-butene/*cis*-2-butene, indicative of intervening S_E2' cleavage. The observation of significant solvent deuterium isotope effects at pH 7.4 of $V_{max}(H_2O)/V_{max}(D_2O) = 2.1$ for *cis*-2-butenyl-2-mercuric chloride (**1**) turnover and $V_{max}(H_2O)/V_{max}(D_2O) = 4.9$ for ethylmercuric chloride turnover provides additional support for a kinetically important proton delivery. Finally, the stoichiometric formation of butene and Hg(II) from **1** and methane and Hg(II) from methylmercuric chloride eliminates the possibility of an S_N1 solvolytic mechanism. As the first well-characterized enzymatic reaction of an organometallic substrate and the first example of an enzyme-mediated S_E2 reaction the organomercurial lyase catalyzed carbon–mercury bond cleavage provides an arena for investigating novel enzyme structure–function relationships.

Organomercurial lyase mediates the first of two steps in the microbial detoxification of organomercurial salts (Summers, 1985; Robinson & Tuovinen, 1984; Silver & Kinscherf, 1982; Summers & Silver, 1978). This first step involves protonolysis of the carbon–mercury bond to yield Hg(II) and the corresponding hydrocarbon (eq 1 and 2). In a second step the

$$\text{R-Hg-X} \xrightarrow{\text{organomercurial lyase}} \text{R-H} + \text{Hg(II)} \quad (1)$$

$$\text{Hg(II)} \xrightarrow[\text{NADPH}\quad\text{NADP}^+]{\text{mercuric reductase}} \text{Hg(O)} \quad (2)$$

Hg(II) is reduced to volatile Hg (0) in an NADPH-dependent reaction mediated by mercuric reductase, completing the detoxification (Fox & Walsh, 1982, 1983; Brown, 1985). In the preceding paper we have described the overproduction, purification, and preliminary characterization of cloned bacterial organomercurial lyase (Begley et al., 1986) from *Escherichia coli* (R831) (Schottel, 1978). This enzyme is one of the few known to process an organometallic compound.

The task of organomercurial lyase is by no means trivial given the stability of organomercurial salts toward strong acids (Jensen & Rickborn, 1968) and bases (Makarova & Nesmeyanov, 1967). The mechanism by which the enzyme effects bond cleavage is thus of fundamental interest. In addition, the environmental consequences of heavy-metal pollutants

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