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Dienelactone Hydrolase from *Pseudomonas* sp. Strain B13

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Dienelactone hydrolase (EC 3.1.1.45) catalyzes the conversion of *cis*- or *trans*-4-carboxymethylenebut-2-en-4-olide (dienelactone) to maleylacetate. An approximately 24-fold purification from extracts of 3-chlorobenzoate-grown *Pseudomonas* sp. strain B13 yielded a homogeneous preparation of the enzyme. The purified enzyme crystallized readily and proved to be a monomer with a molecular weight of about 30,000. Each dienelactone hydrolase molecule contains two cysteinyl side chains. One of these was readily titrated by stoichiometric amounts of *p*-chloromercuribenzoate, resulting in inactivation of the enzyme; the inactivation could be reversed by the addition of dithiothreitol. The other cysteinyl side chain appeared to be protected in the native protein against chemical reaction with *p*-chloromercuribenzoate. The properties of sulfhydryl side chains in dienelactone hydrolase resembled those that have been characterized for bacterial 4-carboxymethylbut-3-en-4-olide (enol-lactone) hydrolases (EC 3.1.1.24), which also are monomers with molecular weights of about 30,000. The amino acid composition of the dienelactone hydrolase resembled the amino acid composition of enol-lactone hydrolase from *Pseudomonas putida*, and alignment of the NH_2-terminal amino acid sequence of the dienelactone hydrolase with the corresponding sequence of an *Acinetobacter calcoaceticus* enol-lactone hydrolase revealed sequence identity at 8 of the 28 positions. These observations foster the hypothesis that the lactone hydrolases share a common ancestor. The lactone hydrolases differed in one significant property: the k_{cat} of dienelactone hydrolase was 1,800 min^{-1}, an order of magnitude below the k_{cat} observed with enol-lactone hydrolases. The relatively low catalytic activity of dienelactone hydrolase may demand its production at the high levels observed for induced cultures of *Pseudomonas* sp. strain B13.

Dienelactone hydrolase (EC 3.1.1.45) (Fig. 1) catalyzes a step in the metabolic conversion of chlorocatechols to β-ketoadipate. Enol-lactone hydrolase (EC 3.1.1.24) (Fig. 1) mediates a chemically analogous step in a similar pathway that gives rise to β-ketoadipate from catechol. The chemical similarity of the two hydrolytic reactions raises the possibility that the hydrolases share a common ancestor.

Enol-lactone hydrolases from *Acinetobacter calcoaceticus* and *Pseudomonas putida* have been previously characterized (11, 17, 22), but comparison of these enzymes with dienelactone hydrolase was not possible because the latter enzyme had not been isolated in homogeneous form. Schmidt and Knackmuss (19) separated enzymes with enol-lactone hydrolase and dienelactone hydrolase activities from extracts of 3-chlorobenzoate-grown *Pseudomonas* sp. strain B13. Each enzyme was inactive with respect to the substrate of the other enzyme, and the inactive lactones did not act as competitive inhibitors. Physical and chemical properties of the enzyme were not examined.

Enol-lactone hydrolases from *A. calcoaceticus* and *P. putida* have molecular weights of approximately 30,000, and each polypeptide contains two or three cysteinyl side chains (11, 17, 22). Incubation of enol-lactone hydrolases with *p*-chloromercuribenzoate at concentrations sufficient to modify a single thiol results in total loss of activity, and the inactivation of the *P. putida* enzyme can be reversed by addition of dithiothreitol (23). Thus it appears that the enol-lactone hydrolases contain a cysteinyl side chain that appears to play an important role in the structure and function of the enzyme.

In this report we describe purification to homogeneity of dienelactone hydrolase from *Pseudomonas* sp. strain B13. We present physical and chemical properties of the purified enzyme, which resembles the enol-lactone hydrolases in a number of respects, including an important sulfhydryl side chain.

MATERIALS AND METHODS

Chemicals. Pharmacia provided chromatographic columns and materials used for isoelectric focusing. Ammonium sulfate (ultrapure) was from Schwarz-Mann; DEAE-cellulose (DE 52) was from Whatman; EM Fractogel TSK HW-55S and TSK HW-55F were from E. Merck AG. Protein assay reagents and standards, as well as protein molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration, were from Bio-Rad Laboratories. Protein molecular weight markers for nondenaturing polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co. *cis* and *trans*-dienelactones (19), (+)-muconolactone (16), and enol-lactone (16) were synthesized enzymatically. All other chemicals were of the highest grade available commercially.

Enzyme assays. Published procedures were used for measurement of muconolactone isomerase (16), enol-lactone hydrolase (16), and dienelactone hydrolase (19). The substrate for the dienelactone hydrolase was the *trans* isomer at a concentration of 33 μM. In every case, 1 U of activity was the amount of enzyme that converted 1 μmol of substrate to product in 1 min under assay conditions. Protein concentrations were determined (3, 20) with bovine serum albumin as the standard. Homogeneous dienelactone hydrolase concentrations were measured at 280 nm: an A_{280} of 1.2 corresponded to 1.0 mg ml^{-1}. Molar concentrations of the enzyme were based upon a molecular weight of 28,000.

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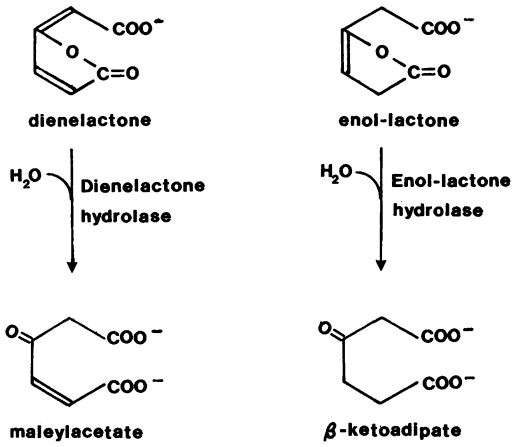

FIG. 1. Reactions catalyzed by dienelactone hydrolase (left) and enol-lactone hydrolase (right). Dienelactone hydrolase acted upon both *cis* and *trans* isomers of its substrate.

Growth and harvest of cells. *Pseudomonas* sp. strain B13 was grown aerobically at 30°C in mineral salts medium supplemented with 3-chlorobenzoate at concentrations that never exceeded 5 mM; the pH was maintained near 6.8 during growth of the 200-liter culture in a New Brunswick IF-250 fermentor. When the turbidity of the culture exceeded 600 Klett units, determined with a red filter, the cells were harvested in a CEPA model Z61 high-speed continuous centrifuge. The recovered cell paste (about 1 kg [wet weight]) was stored at −20°C until use.

Purification of dienelactone hydrolase. Unless noted otherwise, extracts were maintained below 4°C; pH adjustments were made at 20°C. Approximately 250 g of frozen cells was suspended in 1 liter of 50 mM sodium ethylenediamine buffer (pH 7.3) containing 1 mM 2-mercaptoethanol. The cells were lysed by passage through a Dyno-Mill extractor with 0.1-mm lead-free glass beads. The extracted material was stirred vigorously for 1 h, and particulate matter was removed by centrifugation for 30 min at 15,000 × *g*. The resulting preparation (crude extract, Table 1) was treated with 0.4 M ammonium sulfate to disrupt protein aggregation (12), and the ammonium sulfate was removed by repeated dialysis against 10 mM sodium ethylenediamine buffer (pH 7.3) containing 1 mM 2-mercaptoethanol (buffer A). Denatured protein was removed by centrifugation, and the resulting supernatant (dialysate, Table 1) was passed through a DEAE-cellulose column (2.5 by 100 cm) equilibrated with buffer A. After a wash with 1 liter of buffer A, the protein was eluted with 2 liters of buffer A containing sodium chloride in a linear gradient ranging from 0 to 0.2 M; the flow rate was maintained at 50 ml/h. Fractions of 20 ml were collected, and those containing hydrolase activity were pooled (DEAE-cellulose eluate, Table 1). The DEAE-cellulose eluate was fractionated by the addition of crystalline ammonium sulfate; material precipitated by ammonium sulfate between 45 and 60% of saturation was dissolved in 50 mM Tris chloride buffer (pH 7.4) containing 1 mM 2-mercaptoethanol (buffer B) and centrifuged to remove denatured protein. The resulting supernatant (45 to 60% ammonium sulfate fraction, Table 1) was further purified by gel filtration on an EM Fractogel TSK HW-55F column (2.5 by 70 cm) preequilibrated with buffer B. The eluate was collected in 3-ml fractions; those fractions containing the hydrolase were pooled, and solid ammonium sulfate was added to 60% of saturation. The precipitated material was dissolved in buffer B, and the supernatant, after clarification by centrifugation, was collected (gel filtration eluate, Table 1). The gel filtration eluate was dialyzed against 1 liter of 10% saturated ammonium sulfate (pH 7.0) containing 1 mM 2-mercaptoethanol. Crystallization of the enzyme was complete in 4 to 5 days. Crystals were collected by centrifugation and dissolved in 20 mM Tris chloride (pH 7.4) containing 10 μM dithiothreitol. The resulting supernatant after centrifugation was stored as purified enzyme (Table 1).

Analytical techniques. Gel filtration (1) was conducted on a Fractogel TSK HW-55S column (1.6 by 70 cm) equilibrated with 50 mM Tris chloride (pH 7.4) containing 1 mM 2-mercaptoethanol; the column was calibrated for molecular weights with thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B_{12} (1,340). The sample was eluted by gravity at a flow rate of about 20 ml h^{-1}, and the eluate was monitored for A_{280} or by enzyme activity assays. Nondenaturing polyacrylamide gel electrophoresis was performed by procedures described in the Pharmacia manual; i.e., samples containing hydrolase and protein markers in amounts ranging from 1 to 10 μg were run on a 4 to 30% total, 4% cross-linked gradient nondenaturing gel. Thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000), bovine serum albumin (132,000 and 66,000), chicken egg albumin (45,000), and alpha-lactalbumin (14,000) were used as molecular weight standards. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21) was performed with samples containing hydrolase or protein markers in amounts ranging from 5 to 25 μg on a 12% total, 2.7% cross-linked, and 0.1% sodium dodecyl sulfate denaturing gel. Standards included phosphorylase *b* (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,500). Isoelectric focusing was conducted on a 1% agarose gel by procedures outlined in the Pharmacia manual. Protein bands on all gels were visualized by being stained with Coomassie blue R-250.

Amino acid analysis and NH_2-terminal amino acid sequencing. Amino acid compositions were determined on a Dionex 500 amino acid analyzer; 25-μg samples of purified hydrolase were desalted and lyophilized. Samples were subsequently

TABLE 1. Purification of dienelactone hydrolase from *Pseudomonas* sp. strain B13

Step	Vol (ml)	Total protein (mg)	Total activity (U)	Recovery (%)	Sp act (U mg^{-1})	Purification (fold)
Crude extract	1,000	10,000	27,000	100	2.7	1.0
Dialysate	1,200	6,000	27,500	102	4.6	1.7
DEAE-cellulose eluate	185	900	19,500	73	21.2	7.9
45–60% Ammonium sulfate fraction	13	400	15,500	57	34.6	12.8
Gel filtration eluate	13	300	13,000	48	40.0	14.8
Crystallization					65.0	24.1

hydrolyzed at 105°C in 6 N HCl in vacuo for 20, 48, 72, and 110 h. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrochloric acid hydrolysis of the sample (7, 14). Tryptophan was determined after hydrolysis of the sample with 3 N mercaptoethanesulfonic acid (8, 9). The NH_2-terminal amino acid sequence of the desalted, lyophilized hydrolase was determined by automated Edman degradation with an Applied Biosystems model 470A gas-phase protein sequencer; phenylthiohydantoin derivatives were identified by high-performance liquid chromatography on an Altex Ultrasphere ODS-PTH column (13).

Inactivation by *p*-chloromercuribenzoate. Stoichiometric inactivation of dienelactone hydrolase was observed by incubation of 25 nmol of purified enzyme in 1 ml of 100 mM sodium phosphate (pH 7.0; 25 μM) containing *p*-chloromercuribenzoate in concentrations ranging in 5-μM increments from 0 to 50 μM at 25°C. Samples of 1 μl were taken from each mixture after different intervals up to 60 min and suitably diluted for enzyme assays. After incubation of the samples for 60 min, identical mixtures were incubated with 1 mM dithiothreitol for an additional 60 min at 25°C and samples were removed for determination of enzyme activity.

***p*-Chloromercuribenzoate titrations.** Quantitative titration of thiol groups in dienelactone hydrolase with *p*-chloromecuribenzoate was performed by procedures established by Boyer (2). Purified enzyme (10 nmol) in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0, 25°C) was incubated with different amounts of *p*-chloromercuribenzoate; an equal amount of the sulfhydryl reagent was added to 1 ml of phosphate buffer containing no protein. After 15 min, the increment in the absorbance of the two solutions was determined. Attempts to perform a similar titration in 0.33 M acetate (pH 4.6) were unsuccessful because the enzyme precipitated at this pH.

RESULTS

Purification of dienelactone hydrolase. A 24-fold purification (Table 1) with 48% recovery from the crude extract yielded an enzyme that was judged to be homogeneous by polyacrylamide gel electrophoresis and isoelectric focusing. There was no increase in specific activity after successive crystallizations, and the specific activity of the pure protein was judged to be 65 U/mg of protein. This value is lower than the value (205.5 U/mg of protein) reported by Schmidt and Knackmuss (19) for the partially purified enzyme. Enzyme assays were conducted under virtually identical conditions, so there is no clear reason for the discrepancy in specific activities. Purified dienelactone hydrolase stored in 20 mM Tris hydrochloride (pH 7.4) containing 10 μM dithiothreitol was stable for over 6 months at 4°C. The enzyme also was converted to microcrystals by the addition of ammonium sulfate to 30% of saturation; the microcrystals were stable for more than 1 year at 4°C. The enzyme can be crystallized from citrate and phosphate at pH 6 in a form suitable for high-resolution X-ray diffraction studies (15).

Physical and chemical properties of dienelactone hydrolase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of denatured dienelactone hydrolase in the presence of 2-mercaptoethanol revealed a single protein band with a molecular weight of 28,000. The molecular weight of the enzyme was estimated to be 28,500 by both gel filtration and nondenaturing polyacrylamide gel electrophoresis. Thus the enzyme appears to be a monomer with a molecular weight of about 28,000; this conclusion is consistent with the asymmetric unit cell of the enzyme crystal as determined by X-ray diffraction (15). Isoelectric focusing of the enzyme gave rise to a single protein band with an isoelectric point of 5.2 at 25°C. The amino acid composition (Table 2) revealed 2 half-cysteine residues in a protein with 252 amino acid residues. The NH_2-terminal amino acid sequence of dienelactone hydrolase was determined to be Met-Leu-Thr-Glu-Gly-Ile-Ser-Ile-Gln-Ser-Tyr-Asp-Gly-X-Thr-Phe-Gly-Ala-Leu-Val-Gly-Ser-Pro-Ala-Lys-Ala-Pro-Ala-X-Val-Ile-Val-Ile-X-Gln-X-Ile.

TABLE 2. Amino acid composition of dienelactone hydrolase from *Pseudomonas* sp. strain B13

Amino acid	No. of residues	
	Determined	Predicted (per 27,300 Da)[a]
Cys	2.1[b]	2
Asx	16.7[c]	17
Thr	7.9[d]	8
Ser	14.2[d]	14
Glx	31.9[e]	32
Pro	15.9	16
Gly	24.9	25
Ala	33.4	33
Val	18.2	18
Met	4.2	4
Ile	7.0	7
Leu	22.7	23
Tyr	12.1	12
Phe	10.1	10
His	7.2	7
Lys	9.5	10
Arg	10.1	10
Trp	3.9[f]	4

[a] Da, Daltons.
[b] Determined as cysteic acid after performic acid oxidation.
[c] Sum of values for aspartate and asparagine.
[d] Extrapolated to zero time hydrolysis.
[e] Sum of values for glutamate and glutamine.
[f] Determined after hydrolytic treatment with 3 N mercaptoethanesulfonic acid.

Kinetic properties of dienelactone hydrolase. Measurement of the dependence of the initial velocity of the hydrolase on pH revealed a bell-shaped curve, indicating that the enzyme is active from pH 5 to 9, with optimal activity at pH 7.5. As reported by Schmidt and Knackmuss (19), the hydrolase acts on both *cis* and *trans* isomers of the dienelactone, with K_ms of 400 and 15 μM, respectively. The k_{cat} observed with each dienelactone was 1,800 min^{-1}. The enzyme exhibited no activity with (+)-muconolactone or β-ketoadipate enol-lactone. There was no indication that the purified enzyme required coenzymes or specific ions for activity, and the absorption spectrum of the enzyme can be attributed entirely to its amino acid side chains.

Inactivation of dienelactone hydrolase by *p*-chloromercuribenzoate. Dienelactone hydrolase was rapidly inactivated by equimolar amounts of *p*-chloromercuribenzoate (Fig. 2). Maximum inactivation was attained after 15 min, and the inactivation was completely reversed by dithiothreitol. The enzyme maintained full activity when incubated in the absence of sulfhydryl reagents.

***p*-Chloromercuribenzoate titration of sulfhydryl residues.** The results of titration of dienelactone hydrolase with *p*-chloromercuribenzoate are shown in Fig. 3. *p*-Chloromercuribenzoate reacted with 0.94 sulfhydryl group per mol of enzyme, and the molar absorptivity of the mercaptide formed was calculated to be 9.9×10^3 M^{-1} cm^{-1} at 250 nm. This value compares with a molar absorptivity at 250 nm of 7.6×10^3 M^{-1} cm^{-1} obtained for the mercaptide with

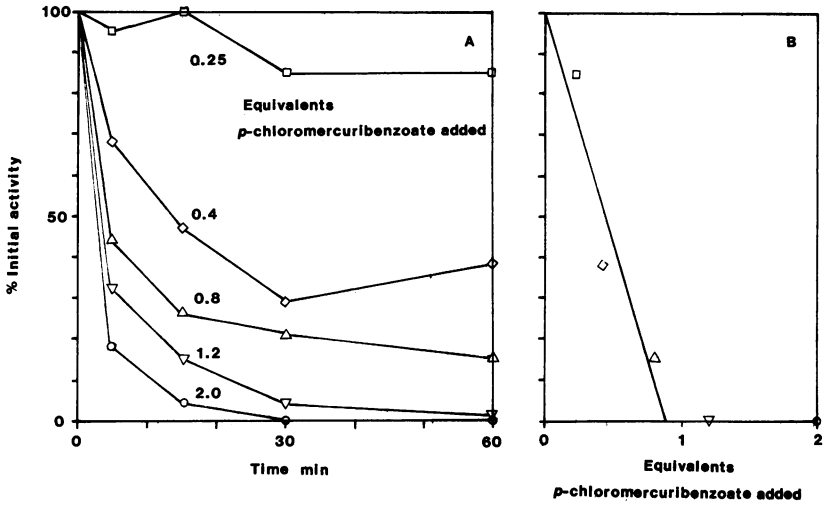

FIG. 2. Inactivation of dienelactone hydrolase by stoichiometric amounts of *p*-chloromercuribenzoate. The enzyme (25 μM) was incubated with *p*-chloromercuribenzoate added at the indicated equivalents. (A) The remaining activity was monitored over 1 h. (B) Approximately 1 equivalent of *p*-chloromercuribenzoate was sufficient to inactivate the enzyme.

cysteine (2). Reaction of the sulfhydryl group of the enzyme with *p*-chloromercuribenzoate followed second-order kinetics, with a rate constant of 2,500 M^{-1} min^{-1}, the same order of magnitude as the constants determined with other proteins (2).

DISCUSSION

Purification of dienelactone hydrolase is a relatively simple procedure because the enzyme accounts for more than 6% of the soluble protein in fully induced cultures of *Pseudomonas* sp. strain B13. Demand for synthesis of large amounts of the enzyme may be necessitated by its low activity: the k_{cat} of 1,800 min^{-1} observed with *cis*- and *trans*-dienelactones with dienelactone hydrolase was an order of magnitude lower than the k_{cat} of about 13,400 min^{-1} calculated for purified enol-lactone hydrolases (11, 17, 22). Furthermore, the dienelactone hydrolase exhibited a relatively high K_m of 400 μM with *cis*-dienelactone, 25-fold higher than the K_m observed with the *trans* isomer. Substantial quantities of the hydrolase may be required for efficient metabolism of the *cis* isomer.

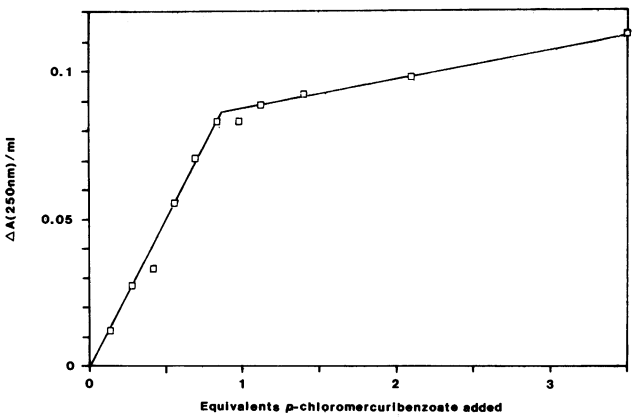

FIG. 3. Titration of sulfhydryl groups in dienelactone hydrolase with *p*-chloromercuribenzoate. Increasing increments of *p*-chloromercuribenzoate were added to 10 nmol of enzyme in 1 ml of buffer, and the increase in A_{250} was measured.

The physical and chemical evidence indicates that the dienelactone hydrolase is a monomer with a molecular weight of about 28,000. Each monomer contains two cysteinyl side chains, one of which is readily titrated by *p*-chloromercuribenzoate. Modification of this sulfhydryl group inactivates the enzyme, and the inactivation is readily reversed by dithiothreitol, so it appears that the titratable sulfhydryl group lies at or near the active site of the enzyme. The remaining cysteinyl group cannot be involved in a disulfide bond because the enzyme exists as a monomer. This cysteinyl side chain may be unreactive, because it lies in a hydrophobic region within the protein.

The physical and chemical properties of dienelactone hydrolase resemble those of bacterial enol-lactone hydrolases. For example, *P. putida* enol-lactone hydrolase is a monomer with a molecular weight of about 28,000. Comparison of the amino acid composition of this enzyme with that of the dienelactone hydrolase by the method of Marchalonis and Weltman (10) gave an SΔQ of 56, near the value of 50, which they found to invariably correspond to sequence homology evident at the level of the amino acid sequence. The enol-lactone hydrolase from *P. putida* contains three cysteinyl side chains; one of these is readily and reversibly titrated by *p*-chloromercuribenzoate (23). As observed with dienelactone hydrolase, equimolar amounts of the sulfhydryl reagent completely inactivated enol-lactone hydrolase, suggesting that the modified sulfhydryl group may be important for activity.

Alignment of the NH_2-terminal amino acid sequences of the hydrolases reveals a level of sequence similarity suggesting a common evolutionary origin for dienelactone and enol-lactone hydrolases. In 28 compared positions, eight identical residues were found when the NH_2-terminal amino acid sequence of dienelactone hydrolase was aligned with the corresponding sequence of enol-lactone hydrolase I from *A. calcoaceticus* (Fig. 4). This extent of sequence similarity is striking, considering the amount of sequence divergence that has occurred within the enzyme family of enol-lactone hydrolases. For example, enol-lactone hydrolases I and II from *A. calcoaceticus* possess only six identical residues in

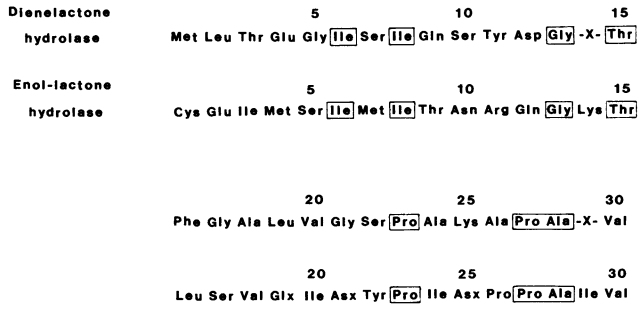

FIG. 4. Comparison of the NH_2-terminal amino acid sequence of dienelactone hydrolase determined in this investigation with the corresponding sequence of enol-lactone hydrolase I from *A. calcoaceticus* (22).

the first 30 positions of their NH_2-terminal amino acid sequences (22). In view of the extensive sequence divergence that has occurred among the hydrolases, the most enduring record of a possible common evolutionary origin may emerge from comparison of sequences lying near the titratable cysteines. These sequences may have been constrained against the extensive divergence that took place at other regions in the proteins.

The most complete evidence concerning evolutionary relationships among the hydrolases will come from comparison of the DNA sequences of their structural genes. A substantial step toward this goal was made possible by isolation of the 3-chlorobenzoate catabolic plasmid pAC25 (4). Although derived from a different biological source, this plasmid closely resembles the plasmid pWR1 (18), which encodes the dienelactone hydrolase from *Pseudomonas* sp. strain B13. A subclone of pAC25 containing a 4.3-kilobase-pair *Bgl*II fragment was shown to contain the structural genes for the degradation of chlorocatechol (6) (Fig. 1). As described elsewhere (5), a 1.9-kilobase-pair *Pst*I-*Bgl*II segment of the *Bgl*II fragment was sequenced and shown to code for a dienelactone hydrolase which closely resembles the dienelactone hydrolase from *Pseudomonas* sp. strain B13.

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