Nucleotide sequence of the structural gene encoding a 2-haloalkanoic acid dehalogenase of *Pseudomonas putida* strain AJ1 and purification of the encoded protein

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The nucleotide sequence of a gene encoding an L-2-haloalkanoic acid halidohydrolase from *Pseudomonas putida* strain AJ1 was determined. The ORF (*hadL*) codes for a polypeptide of 227 amino acids (*M*_r 25687) which has significant homology to two other L-2-haloalkanoic acid halidohydrolases of *Pseudomonas* sp., Dehcl and Dehcll; these show 38% and 51% amino acid identity respectively to HadL. All three enzymes produce products of an opposite optical configuration to that of the substrates. Comparison of the three sequences shows several highly conserved motifs which indicate the possible position of the enzyme active site. The enzyme was purified to homogeneity and appears to exist as a tetramer.

Introduction

Halogenated aliphatic hydrocarbons are a significant group of environmental pollutants and dehalogenation is an important step in their degradation and mineralization. A number of micro-organisms have been isolated which are capable of degrading such compounds (Ghosal *et al.*, 1985; Chaudhry & Chapalamadugu, 1991). Most studies on the genetic basis of such degradation have concentrated on the haloalkanes (Janssen *et al.*, 1989; La Roche & Leisinger, 1990) and haloalkanoic acids.

Bacteria with haloalkanoic acid halidohydrolases appear to be readily isolated from the environment (Hardman & Slater, 1981) and include several strains of *Pseudomonas* spp. (Goldman *et al.*, 1968; Little & Williams, 1971; Slater *et al.*, 1979; Motosugi *et al.*, 1982; Klages *et al.*, 1983; Tsang *et al.*, 1988). These enzymes act by hydrolysing the haloacid (each enzyme is capable of degrading a range of substrates) to yield inorganic chloride. The optical configuration of the products

appears either to be inverted or to be maintained and mechanisms for reactions have been suggested by Weightman *et al.* (1982).

Such bacteria often appear to produce more than one dehalogenase enzyme, specific for either a single stereoisomer of the chemical or active against both isomers (Weightman *et al.*, 1982; Leigh *et al.*, 1988). Some strains appear to encode isofunctional dehalogenase enzymes (Schneider *et al.*, 1991), suggesting that differential regulation of the coding genes or different mechanisms of reaction may optimize the degradative activity.

Pseudomonas putida strain AJ1 was isolated from a soil sample which had previously been exposed to 2-monochloropropionic acid (MCPA; ICI Biological Products, Billingham, UK), a chemical used in the manufacture of a number of herbicides. AJ1 appeared to produce two enzymes, each responsible for the degradation of a single stereoisomer of MCPA. The enzyme with D-MCPA dehalogenase activity (HadD) was purified and shown to exist as a tetramer which is able to degrade a range of short-chain D-2-halocarboxylic acids (Smith *et al.*, 1990). The N-terminal sequence was determined and used to design a single oligonucleotide probe which was used to probe a gene library of AJ1 DNA. A single DNA fragment of 10 kb was isolated (in plasmid pTB281; see Fig. 1) which encoded the enzymes for the degradation of

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Abbreviation: MCPA, monochloropropionic acid.

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both D-MCPA and L-MCPA (HadD and HadL respectively; Barth *et al.*, 1992). In each case the MCPA is hydrolysed to yield lactate with the release of chloride ions (ICI Biological Products, personal communication). The *hadD* gene was sequenced and shown to encode a polypeptide of 300 amino acids (Barth *et al.*, 1992). This appears to show no homology to two genes recently cloned from *P. putida* strain CBS3, *dehC1* and *dehC2*, products of which catalyse the reaction of L-2-monochloropropionic acid to D-lactate (Schneider *et al.*, 1991).

In this paper we report the DNA sequence of *hadL* and describe the purification of the encoded protein.

Methods

Bacterial strains and plasmids. DNA manipulations were performed in *Escherichia coli* JM83 (Yanisch-Perron *et al.*, 1985). The plasmid pUC18 was used as a vector for DNA sequencing (Yanisch-Perron *et al.*, 1985); pTB281 contains the 10 kb fragment of AJ1 DNA (Fig. 1); expression vector pGBT30, provided by Dr G. Jagura-Burdzy, contains a pMB1 replicon, *lacI^q*, and the *tac* promoter upstream of a pUC18 multiple cloning site (Jagura-Burdzy *et al.*, 1991). Plasmids constructed during the course of this work are shown in Fig. 1. Cells were grown at 37 °C in L-broth or on L-agar, supplemented when necessary with penicillin (100 µg ml⁻¹ in LB, 300 µg ml⁻¹ in LA). For induction of the *tac* promoter IPTG was added to 0.5 mM 3 h prior to harvesting the cells.

DNA manipulations. Plasmid DNA was isolated by the alkaline-SDS method of Birnboim & Doly (1979). Restriction enzymes and other DNA-modifying enzymes were purchased from Northumbria Biologicals or Bethesda Research Laboratories and used according to the manufacturer's instructions. Agarose gel electrophoresis was carried out by standard procedures (Sambrook *et al.*, 1989). DNA fragments for cloning were isolated by electrophoresis on a 1% (w/v) TAE agarose gel (Sambrook *et al.*, 1989) and purified using the Gene-clean kit from Bio101.

DNA sequence analysis. DNA sequencing by the chain-termination method (Sanger *et al.*, 1977) was carried out on double-stranded plasmid DNA template using the Sequenase kit supplied by United States Biochemical Corp. (USB). Template DNA was purified by CsCl/ethidium bromide density-gradient centrifugation. Primers were either sequencing primer or reverse sequencing primer from USB, or synthetic 16-mer oligonucleotides synthesized by Alta Bioscience. Sequencing reactions were resolved by electrophoresis on 8% (w/v) polyacrylamide urea gels.

Nucleotide and amino acid sequences were analysed using the UWGCG programs (Devereux *et al.*, 1984).

Protein purification. Bacteria containing the overexpressing plasmid pHAD13 (Fig. 1) were grown overnight in 500 ml L-broth. Cultures were then diluted 1 in 2 and IPTG added to 0.5 mM. After a further 3 h the cells were harvested, resuspended in sonication buffer (20 mM-Tris/HCl pH 8.0, 10 mM-β-mercaptoethanol, 10%, v/v, glycerol, 1 mM-phenylmethylsulphonyl fluoride, 1 mM-EDTA), and sonicated over 10 min (30 s bursts, 30 s cooling periods). The sonicate was centrifuged at 40000 r.p.m. for 1 h, and the supernatant was removed and stored at -70 °C. Extract was applied to a DEAE-Sepharose anion-exchange column equilibrated with 0.05 mM-Tris/HCl pH 7.0 and 0.25 M-NaCl. Protein was eluted in the same buffer. Samples were then applied to an

Anagel-TSK G3000SW 7.5 mm × 60 cm gel filtration column equilibrated with 25 mM-potassium phosphate buffer (pH 7.4), and run on a modular Gilson HPLC system controlled by an IBM PC. Protein samples were analysed on a standard SDS-polyacrylamide gel (Laemmli, 1970) and stained with Coomassie brilliant blue R250. When necessary, proteins were concentrated either by membrane filtration using a Filtron Microsep centrifugal microconcentrator (Flowgen), or by precipitation with 4 vols methanol/acetone (1:1, v/v).

Qualitative assay of dehalogenase activity. Dehalogenase activity of protein samples in a non-denaturing gel was detected by immersion in a solution of 50 mM-MCPA followed by detection of released Cl⁻ ions by precipitation of silver chloride. Duplicate samples were electrophoresed on a standard protein gel (Laemmli, 1970) except that 0.1 mM-DTT was used in place of 10% (w/v) SDS, and all buffers were Tris/H₂SO₄. Samples were not boiled prior to loading, contained no bromophenol blue or other dye, and were electrophoresed at 4 °C until marker dye in an adjacent well had reached the bottom of the gel. Half the gel was stained with Coomassie brilliant blue R250. The other half was immersed in 50 mM-DL-MCPA in 0.2 M-Tris/H₂SO₄ pH 8.0 at room temperature for 40 min. It was then washed thoroughly with water and then placed in 0.1 M-AgNO₃ for a few minutes until a precipitate of AgCl₂ became visible on the gel. This demonstrates the presence of Cl⁻ ions, which correspond to the position of any protein in the gel that is able to dechlorinate the MCPA. The gel was fixed in 5% (v/v) acetic acid for 15 min and then vacuum-dried.

Quantitative assay of dehalogenase activity. Release of halide ions was detected by titration against silver nitrate on a Mettler DL21 automatic titrator: 1 ml of a buffered solution of 100 mM-substrate was titrated with 10 ml 10 mM-AgNO₃ and the end-point determined by potentiometric analysis.

Determination of the optical configuration of lactate product. The presence of D- or L-lactate was assayed by the principle described by Noll (1984). Assay mixtures contained 810 µl H₂O, 30 µl 50 mM-NAD, 110 µl 1 M-glycine/NaOH pH 10.0, 20 µl DL-MCPA pH 7.2, 30 U of either *Leuconostoc mesenteroides* D-lactate dehydrogenase (Sigma) or rabbit L-lactate dehydrogenase (Boehringer Mannheim), and an aliquot of HadL. The reaction proceeded at 25 °C and the absorbance was measured at 340 nm in a Pye Unicam SP1800 spectrophotometer.

Determination of N-terminal amino acid sequence. Homogeneous samples of protein were analysed on an Applied Biosystems protein sequencer.

Results

Physical mapping of the *hadL* gene

The structural gene *hadL* was known to lie downstream of *hadD* on a 10 kb fragment of DNA cloned from *Pseudomonas putida* strain AJ1, and was suspected to be transcribed in the same direction as *hadD* (Fig. 1; Barth *et al.*, 1992).

The 2.9 kb *Bam*HI fragment containing most of *hadD* and a large downstream portion of DNA was subcloned into the high-copy-number vector pUC18 (Fig. 1). This 2.9 kb fragment was then inserted into the expression vector pGBT30 downstream of the *tac* promoter. Upon SDS-PAGE analysis, a protein of *M_r* 26000 was

Fig. 1. Cloning of *hadL* into an expression vector. The dehalogenase genes were cloned on a 10 kb fragment into the IncQ plasmid vector pTB244 to give pTB281 (Barth *et al.*, 1992). The 2.9 kb *Bam*HI fragment was first cloned into the high-copy-number vector pUC18, and three DNA fragments containing *hadL* were then cloned downstream of the *tac* promoter in the multiple cloning site of pGBT30. The sequencing strategy employed for the sequencing of *hadL* is also shown.

observed in cultures induced with IPTG. A 0.85 kb *Sst*I–*Sst*II fragment and a 0.9 kb *Sst*I–*Eco*RV fragment were also cloned into pGBT30 (pHAD13 and pHAD14; Fig. 1). The *Sst*I site lies very close to the end of *hadD*, and it was hoped that one or both fragments would contain the intact putative *hadL* gene. SDS-PAGE analysis of bacteria containing these plasmids demonstrated the presence of a protein of a size identical to that previously seen from the larger DNA fragment (Fig. 2). This demonstrated that an intact gene lay before the *Sst*II site as the 0.85 kb fragment does not produce a truncated protein. These plasmids also produced a large amount of the M_r 26000 protein (10–15% of total protein) and so

could be used as a source for subsequent purification of the protein.

Purification and identification of *HadL*

The majority of the M_r 26000 protein produced from pHAD13 appeared to be in soluble form, present mainly in the supernatant (fraction I) after sonication, although some remained in the cell debris. Upon anion-exchange chromatography at pH 7.0 the protein eluted very slowly at 0.25 M-NaCl. At this salt concentration most cellular proteins eluted first, leaving the putative *HadL*, an

Fig. 2. Induced (+) and uninduced (–) *E. coli* strains containing the control plasmid pGBT30 with pHAD11, pHAD13 and pHAD14. The overexpressed polypeptide running at M_r 26000 is the putative HadL. It can be seen that pHAD13 does not produce a truncated protein.

unidentified protein and a low background of protein eluting later. HadL was estimated to be approximately 60% of total protein after this step (fraction II).

To purify HadL further, the eluent from the anion-exchange column was passed down an HPLC gel filtration column, and fractions obtained in which HadL was the only protein detected by the HPLC UV monitor (fraction III). HPLC gel filtration elution times were calibrated with a set of protein standards. From this it appeared that HadL eluted predominantly as a protein of M_r 79000, with a smaller peak at M_r 21000, suggesting that the protein exists as a tetramer. When the larger species was analysed by SDS-PAGE it ran with an apparent M_r of 26000. Under some conditions homogeneous samples of the protein also appeared to elute as a protein of M_r 52000, presumably the dimer. Furthermore, if the sample was boiled prior to gel filtration the M_r 21000 protein was the only species present. It thus seems likely that HadL exists as a tetramer (as indeed does HadD; Smith *et al.*, 1990), although the data do not completely rule out the possibility that it is a trimer.

The first 30 amino acids of the protein in fraction III were determined and were found to correspond precisely with the predicted sequence shown in Fig. 3, except residues at position 2 (Lys), which was unclear, and position 25 (Cys), which was unidentified.

Fractions II and III were tested on a non-denaturing

protein gel for dehalogenase activity. After soaking the gel in MCPA and then adding silver nitrate, a silver chloride precipitate appeared almost immediately as a single band on the gel in the fraction II sample; no activity was detectable from fraction III. However, staining duplicate lanes with Coomassie blue showed that the band from fraction II with dehalogenating activity ran at precisely the same position as the purified protein obtained from the HPLC purification. The protein sequencing data described above established that this protein was encoded by the gene downstream of *hadD*. The dechlorination test therefore confirms that this gene encodes HadL, although gel filtration under the conditions used results in loss of most of the enzyme activity.

To determine whether HadL hydrolysed L-MCPA to L-lactate or D-lactate the reaction was linked to either L- or D-lactate dehydrogenase. Only in the presence of D-lactate dehydrogenase and HadL was NAD reduced to NADH; no activity was apparent with L-lactate dehydrogenase. This demonstrates that HadL degrades L-MCPA to D-lactate.

Nucleotide sequence of *hadL*

The strategy employed to sequence the region encoding *hadL* is shown in Fig. 1. The region contained a single ORF of 681 bp (Fig. 3) starting from an ATG initiation codon 23 bp downstream of the stop codon of the *hadD* ORF. The predicted amino acid sequence from this start is identical to the N-terminal amino acid sequence obtained from HadL, demonstrating that this is the translational start. The *hadL* ORF has a G + C content of 52 mol%, which is lower than that of the *P. putida* chromosome (60–63%; Normore, 1976) although in the third position of each codon this figure is 61%. This bias is reflected in the codon usage, with codons containing A and T present at a higher frequency than is normally found in *Pseudomonas* sp. (Wada *et al.*, 1991). The ORF terminates 40 bp before the *Sst*II site, known from the studies described earlier to lie after the structural gene.

Upstream of the start codon is a good ribosome-binding site (AAGGAA), but no consensus promoter sequence is apparent. Furthermore, insertion of a transposon immediately upstream of *hadD* in pTB281 (which contains the 10 kb fragment of AJ1 DNA; Fig. 1) abolishes expression of *hadL* (data not shown), suggesting that *hadD* and *hadL* are cotranscribed from a promoter upstream of *hadD*. Approximately 240 bp downstream of *hadL* is a potential transcriptional terminator (Platt, 1986) which contains a stem of seven perfectly matched bases and a loop of nine bases, and is followed by an AT-rich region. Expression studies currently in hand suggest this is indeed a transcriptional

10 SstI 30 50
 AGCGAGGCGCAAACCGATGAGCTCATACAGGTCATCAGCTTGTTCGAATGGATGCTCTCG
 TCGCTCCGCGTTTGGCTACTCGAGTATGTCCAGTAGTCGAACAAGGTTACCTACGAGAGC
 S E A Q T D E L I Q V I S L F Q W M L S

70 90 *hadD stop* 110
 GGACTGGTTTTGAACGTCACACATTTCAAGCAGCAGGCGCTGAAATTAAGCACCTTCCAAAG
 CCTGACCAAACTTGCAGTGTGTAAAGTTCGTCTCGTCCGCGACTTTATTCGTGAAGGTTTC
 G L V L N V T H F K Q Q A L K *

S-D 130 *hadL start* 150 170
 GAAATAACCAATGAAAAACATCCAAGGTATCGTTTTTCGATTGTATGGCAGCTCTACGA
 CTTTATTGGTTACTTTTTGTAGGTTCATAGCAAAAGCTAAACATACCGTGCAGATGCT
 M K N I Q G I V F D L Y G T L Y D

190 210 230
 CGTGCAATTCCGTGGTGCAAGCCTGTGAAGAGGTCTATCCGGGCAAGGCGACGCTATTTTC
 GCACGTAAGGCACCACGTTCCGGACACTTCTCCAGATAGGCCCGGTTCCGCTGCGATAAAG
 V H S V V Q A C E E V Y P G Q G D A I S

250 270 290
 TCGCCTCTGGCGGCAAAAGCAATTGGAATACACCTGGCTCAGGAGCCTCATGGGCCGTTA
 AGCGGAGACCGCCGTTTTTCGTTAACCTTATGTGGACCGAGTCTCGGAGTACCCGGCAAT
 R L W R Q K Q L E Y T W L R S L M G R Y

310 330 350
 CGTGAACCTTTGAGAAAGCAACAGAGGATGCCCTTGCGCTTTACCTGCACGCATCTGGGCTT
 GCACTTGAAACTCTTTCTGTTGTCTCTACGGAACGCGAAATGGACGTGCGTAGACCCGAA
 V N F E K A T E D A L R F T C T H L G L

370 390 410
 GTCGCTCGATGATGAAACCCACCAGCGCCTCAGTGATGCTTATTTGCACCTCACCCCTTA
 CAGCGAGCTACTACTTTGGGTGGTTCGCGGAGTCACTACGAATAAACGTGGAGTGGGGAAT
 S L D D E T H Q R L S D A Y L H L T P Y

430 450 470
 TGCCGATACAGCTGACGCCGTTTCGCCGTTTGAAAGCTGCGGGCCTACCGCTAGGCATCAT
 ACGGCTATGTGCGACTGCGGCAAGCGGCAAACTTTTCGACGCCCGGATGGCGATCCGTAGTA
 A D T A D A V R R L K A A G L P L G I I

490 510 530
 TTCAAATGGTTCTCATTGCTCGATCGAGCAAGTCGTGACTAACTCTGAAATGAATTGGGC
 AAGTTTACCAAGAGTAACGAGCTAGCTCGTTCAGCACTGATTGAGACTTTACTTAACCCG
 S N G S H C S I E Q V V T N S E M N W A

550 570 590
 GTTCGATCAGCTGATCAGCGTCGAGGATGTGCAAGTGTCAAACCTGATAGTCGCGTCTA
 CAAGCTAGTCGACTAGTCGAGCTCCTACACGTTCAAGTTTGGACTATCAGCGCAGAT
 F D Q L I S V E D V Q V F K P D S R V Y

610 630 650
 TAGCCTTGCCGAGAAGCGCATGGGTTTTCCAAAGGAAAACATCCTCTTCGTTTTCGTCAAA
 ATCGGAACGGCTCTTCGCGTACCCAAAAGGTTTCCTTTGTAGGAGAAGCAAAGCAGTTT
 S L A E K R M G F P K E N I L F V S S N

670 690 710
 CGCGTGGGATGCGAGTGCAGCCAGTAACTTTGGTTTTCCCGGTTTGCTGGATCAATCGGCA
 GCGCACCTACGCTCACGTGGTCAATTGAAACCAAAGGGCCAAACGACCTAGTTAGCCGT
 A W D A S A A S N F G F P V C W I N R Q

Fig. 3. Nucleotide sequence of *hadL* (continued on following page).

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      730              750              770
GAACGGCGCGTTTTGATGAGCTGGATGCAAAGCCGACACACGTCGTGCGTAATCTCGCCGA
CTTGCCGCGCAAACACTACTCGACCTACGTTTCGGCTGTGTGCAGCACGCATTAGAGCGGCT
  N  G  A  F  D  E  L  D  A  K  P  T  H  V  V  R  N  L  A  E

      790              810      hadL stop      830
AATGTGGAAGTGGCTGGTTAATTCGCTCGATTAATCCATTGCATCTGGATTGGGAGAATC
TTACAGCTTGACCGACCAATTAAGCGAGCTAATTAGGTAACGTAGACCTAACCCCTCTTAG
  M  S  N  W  L  V  N  S  L  D  *

      850              SstII 870              890
GTCCGCTCAGGGTGAAATCGCGCCGCGGTTTTCCCTGGCGGACATTAGAGCGGAAGATAA
CAGGCGAGTCCCACTTTAGCGCGCGGCCAAAAGGGACCGCCTGTAATCTCGCCTTCTATT

      910              EcoRV              930              950
ATAAGGTGTGCGACGATATCGGCGCCTGTGAAATAGGACCGTAGTCGCACAGTAATAAAC
TATTCACACGCTGCTATAGCCGCGGACACTTTATCCTGGCATCAGCGTGTCAATTATTTG

      970              990              1010
TCAAGTATCCGTGGTTATTTTATGTACGGTTACCTTTCCCGACTTTCATTTGGGCGGGCC
AGTTCATAGGCACCAATAAAATACATGCCAATGGAAAGGGCTGAAAGTAAACCCGCCCGG

      1030              1050      term?      1070
ATCCTCTGGTGCTATCTGATCTGGTACAGCATGGCAATTGTGCTGTATTTCGATTCTGTCG
TAGGAGACCACGATAGACTAGACCATGTCTGACCGTTAACACGACATAAAGCTAAGCAGC

      1090      HpaI              1110              1130
CCCGCCCTTTGGTTAACCTCATTTGGGATCGGCTTACTGATGGGCTTTGGCATGCTTGTC
GGGCGGGAAACCAATTGGAGTAAACCCTAGCCGAATGACTACCCGAAACCGTACGAACAG

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Fig. 3. Nucleotide sequence of *hadL*. The stop codon of *hadD* at position 106 is boxed, as are the start and stop codons of *hadL* (positions 131 and 812). The ribosome-binding site for *hadL* is underlined and the inverted repeat region which is proposed to be the transcriptional terminator downstream of *hadL* is indicated.

terminator, showing *hadD* and *hadL* to be the last two genes in an operon.

Amino acid sequence of *HadL*

The amino acid sequence of *HadL* shows that it is composed of subunits of M_r 25686, closely matching the estimate of M_r 26000 from SDS-PAGE analysis of the protein. It has a deduced isoelectric point of 5.0.

Comparison of the amino acid sequence of *HadD* and *HadL* shows no homology between the proteins, indicating different ancestral origins. However, the sequence of *HadL* is highly homologous to two L-haloalkanoic acid dehalogenases of *Pseudomonas* sp. strain CBS3 (Schneider *et al.*, 1991). These proteins, *DehI* and *DehII*, show 38% and 51% amino acid identity respectively to *HadL*, and show 38% identity between themselves (Fig. 4). The proteins are also almost identical in length. The highest homologies appear to fall in three regions of the proteins, indicated in Fig. 4. The first region is the N-terminal domain, in which all three proteins have a number of hydrophobic residues. The

second and third regions of higher conservation show a number of conserved residues between all three proteins, with relatively high numbers of absolutely conserved Trp, Phe, Tyr and Gln residues. *DehI* has also been purified (Klages *et al.*, 1983), and this will be referred to in the Discussion. *HadL* appears to show no homology to any other protein in the GenEMBL database.

Biochemical characteristics of *HadL*

The enzyme activity of the fraction II preparation was measured at a range of pH values in 1 M-potassium phosphate buffer (pH 7.0–8.0) or in 1 M-glycine/NaOH buffer (pH 9.0–11.0). The optimum pH appeared to lie between 10 and 11; chloride release from MCPA was approximately fivefold greater at pH 10.0 than at pH 7.0. At pH 9.0, chloride was released at approximately $1 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

The activity of *HadL* against 2,3-dichloropropionate (DCPA), 2-monochlorobutyrate (MCBA), 2,3-dibromopropionate (DBPA) and 2-monobromobutyrate (MBBA) was also determined in the same buffers. At pH 9.0,

	0			49
DehcI	VDPIRACVFD	AYGTLLDVNT	AVMKHAHDIG	GCAEELSSLW RQRQLEYSWT
HadL	MKNIQGIVFD	LYGTLYDVHS	VVQACEEVYP	GQGDAISRLW RQKQLEYTWL
DehcII	MQEIRGVVFD	LYGTLCDVHS	VAQLCGQYFP	ERGTEISLMW RQKQLEYSWL
	50			99
DehcI	RTLMGRYADF	WQLTTEALDF	ALESFGLLER	TDLKNRLLDA YHEL SAYPDA
HadL	RSLMGRYVNF	EKATEDALRF	TCTHLGLSLD	DETHQRLSDA YLHLTPYADT
DehcII	RSLMGQYVSF	PQATEDALVF	VCNALNLKLR	EDTRIALCNE YLNIKPYREV
	100			149
DehcI	VGTLGALKAA	GFTTAILSNG	NNEMLRGALR	AGNLTEALDQ CISVDEIKIY
HadL	ADAVRRLKAA	GLPLGIISNG	SHCSIEQVVT	NSEMNAWAFDQ LISVEDVQVF
DehcII	RSALSLRSG	AVPLAILSNG	SAHSIQSVVG	NAGIEHFFSH LISADEVSVS
	150			199
DehcI	KPDP RVYQFA	CDRLDVRPSE	VCFVSSNAWD	IGGAGAFGFN TVRINRINKP
HadL	KPDSRVYSLA	EKRMGF PKEN	ILFVSSNAWD	ASAASNFGFP VCWINRQNGA
DehcII	KPSPAAYELA	EKRLKVVR SK	LLFVSSNAWD	ASGARHFGFQ VCWVNRSRNT
	200			
DehcI	QEYSFAPQRH	QLSSLSEL PQ	LLLRLTQ*	227
HadL	FDELD AKPTH	VVRNLAEM SN	WL.VNSLD*	227
DehcII	FEOLGERPDH	VISGLDEL PN	LLNFASADR*	229

MCBA and MBBA were degraded with approximately 75% of the efficiency that was observed for MCPA (calculated after correcting for spontaneous breakdown). At the same pH value, DCPA was degraded at approximately 30% of the efficiency of MCPA. DBPA was unstable at pH 9.0, but at lower pH values it was degraded at a similar rate to DCPA. No other chemicals were tested.

Discussion

(Leigh *et al.*, 1988) and the enzymes are insensitive to thiol-blocking agents (Little & Williams, 1971). Type 2 enzymes dehalogenate both D- and L-haloacids, again invert the optical configurations of the products (Leigh *et al.*, 1988) and are insensitive to thiol-blocking agents (Motosugi *et al.*, 1982; Weightman *et al.*, 1982). Type 3 enzymes again dehalogenate both D- and L-haloacids, but the products are of the same optical configuration as the substrates and these enzymes are sensitive to thiol-blocking agents (Weightman *et al.*, 1982). Type 4 enzymes are specific for D-haloacids, inverting the optical configuration of the substrate (Leigh *et al.*, 1988), and again are insensitive to thiol-blocking agents (Smith *et al.*, 1990).

The enzyme described in this paper appears to be of the type 1 class. Other enzymes which appear to be of this type include those described by Goldman *et al.* (1968),

Little & Williams (1971), Klages *et al.* (1983), Leigh *et al.* (1988) and Tsang *et al.* (1988). The enzyme isolated from *Pseudomonas* sp. NCIB 9061 (Little & Williams, 1971) was reported to have an M_r of 15000. The enzymes isolated from *P. cepacia* MBA4 (Tsang *et al.*, 1988) and *Pseudomonas* sp. CBS3 (Dehcl; Klages *et al.*, 1983) were reported to have M_r values of 23000 and 28000 respectively, closer to the M_r of 26000 seen for HadL upon SDS-PAGE analysis. However, both of these proteins were reported to exist as dimers, in contrast to HadL, which appears to be a tetramer. Dehalogenase enzymes have been grouped according to their relative electrophoretic mobilities (Tsang *et al.*, 1988) so HadL may fall into a different group in this respect. The enzymes isolated from *P. cepacia* and *Pseudomonas* sp. CBS3 also have pH optima at approximately 9.5, slightly lower than that observed for HadL (although this could be an artefact of different assay and buffer conditions).

The enzyme from *Pseudomonas* sp. NCIB 9061 was little affected by a range of inhibitors, indicating that thiol and free amino groups, and Ser and Tyr residues, were unlikely to be involved in the active site of the enzyme (Little & Williams, 1971). Since activity was lost upon oxidation and/or halogenation with iodine, which reacts primarily with the amino acid residues Trp, His, Tyr and Cys (with increasing efficiency), Little & Williams (1971) suggested that His was the residue in the active site of the enzyme (although they did not rule out Trp). From the sequence data of HadL it can be seen that there are no His residues in the three regions of high homology to Dehcl and DehcII. There is a conserved His at position 210, however, and DehcII and HadL share a conserved His in the first region of high identity. A number of Trp residues are also conserved between proteins in the regions of high identity.

It has also been suggested that the carboxy group of the acid substrate interacts with a basic group on the enzyme to bring about the correct steric conformation. HadL has a pH optimum of about 10.5. Its primary substrate, MCPA, has a pK_a of 2.83 so would be completely dissociated in this pH range, permitting interaction with a basic group. In the second region of high identity between Dehcl, DehcII and HadL there are two absolutely conserved Arg residues (pK_a 12.48), and a third conserved Arg is found at position 195. One or more of these could be involved in the formation of a positively-charged pocket to which the carboxy group may bind. From these homologies it should be possible to study further the enzyme action and the mechanism of the reaction by mutation of the encoding DNA in these regions.

Since this paper was submitted for publication, a haloacid dehalogenase from *Xanthobacter autotrophicus* GJ10 has been characterized and the nucleotide

sequence of the gene determined (van der Ploeg *et al.*, 1991). This protein has a high degree of homology to HadL, Dehcl and DehcII.

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