A bacterial esterase is homologous with non-haem haloperoxidases and displays brominating activity

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Screening GenBank indicated that an esterase from *Pseudomonas fluorescens* had high sequence similarity with bacterial non-haem haloperoxidases. However, this homology was limited to two distinct domains of the published esterase sequence. As errors in the published sequence were suspected, the esterase gene was sequenced again. The revised sequence displayed between 40 and 50% identical amino acids with the haloperoxidases, but distributed along the whole sequence. In addition to the structural homologies with haloperoxidases, the esterase also displayed functional homology. The recombinant esterase, purified from *Escherichia coli* cells, was capable of both ester hydrolysis and halogenation, as detected *in situ* by the formation of bromophenol blue or spectrophotometrically by the bromination of monochlorodimedon. The esterase is thus a bifunctional enzyme. The sequence analysis and the biochemical investigations show that the esterase belongs to the haloperoxidase family. It also possessed, however, a typical feature of serine-hydrolases, namely the consensus motif Gly-X-Ser-X-Gly around the active serine of the catalytic triad. By alignment of the esterase with different serine-hydrolase sequences, it was possible to identify the other two residues of the triad. The triad comprised the residues Ser95, Asp223 and His252. Interestingly, a structurally equivalent catalytic triad was also identified in the sequences of all bacterial non-haem haloperoxidases, in highly conserved domains. The presence of a catalytic triad in haloperoxidases is expected to be important in the mechanism of halogenation.

Keywords: esterase, non-haem chloroperoxidase, *Pseudomonas fluorescens*, catalytic triad.

INTRODUCTION

Haloperoxidases are enzymes able to halogenate a wide variety of substrates in the presence of hydrogen peroxide and halide ions. The cloning and sequence analysis of four haloperoxidases from bacterial sources have pointed to a high degree of homogeneity. The four enzymes analysed are: bromoperoxidases BPO-A1 (Pelletier *et al.*, 1994) and BPO-A2 (Pfeifer *et al.*, 1992), both from *Streptomyces aureofaciens* ATCC 10762, a producer of 7-chlorotetracycline; chloroperoxidase CPO-P from *Pseudomonas pyrro-*

cinia, a producer of the chlorinated antifungal antibiotic pyrrolnitrin (Wolfframm *et al.*, 1993); and chloroperoxidase CPO-L from *Streptomyces lividans*, which is not known to produce a halogenated compound (Bantleon *et al.*, 1994). They displayed high similarity to each other with regard to both structural and catalytic features.

These four enzymes differ considerably from previously characterized haloperoxidases such as (i) various well-investigated haem-type haloperoxidases (Dawson & Sono, 1987), for example the BPO from the green alga *Penicillus capitatus* (Manthey & Hager, 1989), and (ii) the metal-containing haloperoxidases, for example the vanadium-containing BPO from the brown alga *Asco-phyllum nodosum* (De Boer & Wever, 1988), in that they do not contain a prosthetic group or a metal ion to perform the halogenation reaction. Therefore, the bacterial non-haem haloperoxidases form a totally distinct

Abbreviations: BPO, bromoperoxidase; CPO, chloroperoxidase; MCD, monochlorodimedon; pNPA, 4-nitrophenylacetate; RBS, ribosome-binding site; RP, reverse primer; UP, universal primer.

The GenBank accession number for the sequence reported in this paper is U12537.

enzyme family, probably with unique features involved in their catalytic mechanism.

Recently, a major contribution to the understanding of the structure–function relationships among these haloperoxidases has resulted from screening databases for sequences similar to haloperoxidases. This revealed that a protein described as an aryl-esterase from *Pseudomonas fluorescens* (accession number gp/D12484/; Choi *et al.*, 1990) displays surprisingly high homology with CPO-L from *S. lividans* (32% identical amino acids). However, careful comparison of the esterase and chloroperoxidase sequences led us to suppose that there were mistakes in the published esterase gene sequence. When three regions of the esterase gene were translated in the two other frames, the overall homology between the deduced protein and CPO-L increased from 32 to 50%. In order to clarify this intriguing result and to further investigate the relationship between the esterase of *P. fluorescens* and the bacterial haloperoxidases, a plasmid bearing the esterase gene was obtained from O. J. Yoo (Taejon, Korea).

In this study, we report the new sequence analysis of the esterase gene and of the deduced protein, allowing sequence comparisons with the bacterial haloperoxidases. Production of the esterase from *Escherichia coli* cells enabled us to perform comparative studies with the four bacterial haloperoxidases referred to above. Furthermore, purification of the esterase and of CPO-P allowed us to carry out biochemical investigations and to compare more precisely the catalytic features of the two enzymes.

METHODS

Materials. Monochlorodimedon (MCD), phenol red, hydrogen peroxide (30%, v/v) 4-nitrophenyl acetate (pNPA), 2-naphthyl acetate, phenyl acetate, Diazo Blue B (tetraazotized *o*-dianisidine) and tributyrin were purchased from Sigma. X-Gal, IPTG, ampicillin, restriction endonucleases, calf intestinal alkaline phosphatase (CIAP) and bacteriophage T4 DNA ligase were obtained from Boehringer Mannheim. Low melting point Seaplaque GTG agarose was from Biozym Diagnostic. Bio-Rad Protein Assay was from Bio-Rad.

Strains, plasmids and growth conditions. *E. coli* JM109 (Yanisch-Perron, 1985) was used as host for expression of the chloroperoxidase and esterase genes from *Pseudomonas* strains. A 1.5 kb *EcoRI* DNA fragment bearing the *cpoP* gene from *Pseudomonas pyrocinia* on the plasmid pHM321 (Wolfframm *et al.*, 1993) was inserted into the *EcoRI* site of the high-copy-number plasmid pBTac1 (Brosius *et al.*, 1981), with *cpoP* expression under the control of the inducible *tac* promoter, resulting in pIP50. The esterase gene from *Pseudomonas fluorescens* SIK W1 (Choi *et al.*, 1990) was kindly provided by O. J. Yoo (Department of Life Science, Taejon, Korea), on the plasmid pUE1251, which consisted of a 1.2 kb *PstI* DNA fragment inserted into the *PstI* site of pUC19 (Yanisch-Perron, 1985), with the esterase expression under the control of the *lac* promoter. pIP50 and pUE1251 were used to transform JM109 cells and transformants were selected on agar plates containing 100 µg ampicillin ml⁻¹, 40 µg X-Gal ml⁻¹ and 0.2 mM IPTG. *E. coli* JM109 cells containing pIP50 or pUE1251 were grown at 37 °C in Luria-Bertoni broth supplemented with ampicillin until the early exponential phase (OD₆₀₀ 0.5). Gene expression was then induced by adding 0.1 mM IPTG to the cultures, followed by further incubation for 3 h at 30 °C.

Preparation and analysis of protein samples. Crude cell extracts were prepared by resuspending *E. coli* cells in 100 mM sodium acetate pH 5.5 to carry out bromoperoxidase assays, or in 10 mM potassium phosphate buffer pH 7 to perform esterase assays. Cells were disrupted by sonication with a sonifier (Sonicator W-385, Ultrasonics microtip, 6 × 30 s pulse 50% s⁻¹ at 4 °C) and cell debris removed by centrifugation to give a clear lysate. Protein concentrations were determined at 595 nm using the method of Bradford (1976) with bovine serum albumin as standard. Enzyme expression was monitored by loading crude extracts on a 12% (w/v) SDS-PAGE gel using the discontinuous buffer system of Laemmli (1970). Gels were stained for proteins with Serva Blue R (Serva Feinbiochemica). Proteins from the low-*M_r* calibration kit from Pharmacia were used as standard. The *M_r* of the esterase was estimated from a plot of log *M_r* versus mobility using the standard proteins.

Analytical isoelectric focusing was performed with a PhastGel IEF as recommended by Pharmacia, containing ampholytes in the pH range 3–9. The pI of the esterase was calculated from a plot of pI versus mobility using standard proteins in the pI range 4.6–9.6 (IEF Standards, Bio-Rad).

Enzyme assays. Esterase production by cells containing pUE1251 or pIP50 was directly tested in an agar diffusion plate assay: agar plates were overlaid by a soft agar containing an emulsion of 1% (v/v) tributyrin (Kugimiya *et al.*, 1986). Lipolytic activity was indicated by a halo of hydrolysis around the colonies.

Brominating and esterase activities were assayed *in situ* on native 10% (w/v) PAGE gels. Brominating activity was detected by the conversion of the substrate phenol red to bromophenol blue (Loo *et al.*, 1964). Esterase activity was detected *in situ* using the procedure described by Rosenberg *et al.* (1975), with phenyl acetate or 2-naphthyl acetate as substrates and Diazo Blue B as coupling agent.

Brominating activity was also determined spectrophotometrically with the substrate MCD ($\epsilon = 19.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) at 290 nm (Hewson & Hager, 1980; Pfeifer *et al.*, 1992). One unit bromoperoxidase activity was defined as the amount of enzyme catalysing the formation of 1 µmol monobromomonochlorodimedon min⁻¹ at 25 °C. Esterase activity was measured in a colorimetric assay in 10 mM potassium phosphate buffer pH 7 using pNPA (1 mM dissolved in DMSO) as substrate (Donnelly & Crawford, 1988). The amount of 4-nitrophenol released by the esterase activity was determined at 410 nm ($\epsilon = 16.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). One unit of activity was defined as the amount of enzyme releasing 1 µmol 4-nitrophenol min⁻¹ at pH 7 and 25 °C. Determination of the pH optimum was carried out in 10 mM potassium phosphate buffer in the pH range 4–9.

Purification of the esterase and CPO-P from *E. coli*. Crude cell extract from a 500 ml culture (5 g wet weight cells) was diluted 1:20 in water and fractionated by anion-exchange chromatography (Mono Q, Pharmacia). The column was equilibrated with 10 mM potassium phosphate pH 7. Proteins were eluted with a linear gradient of 0 to 1 M sodium chloride in 10 mM potassium phosphate buffer pH 7. Fractions of 1 ml were collected and assayed for protein (*A*₂₈₀) and esterase (*A*₄₁₀) activity. Fractions containing at least 50% of the esterase activity of the most active fraction were pooled and concentrated in an ultrafiltration cell (YM-10 membrane, Amicon). A similar protocol was used to purify CPO-P from crude extracts of cells containing pIP50. Fractions of 1 ml collected from the Mono Q column were assayed for protein (*A*₂₈₀) and bromoperoxidase (*A*₂₉₀) activity. Fractions containing CPO-P were combined and concentrated by ultrafiltration.

DNA manipulation. Plasmid DNA was isolated from *E. coli*

strains using an alkaline lysis procedure followed by spermine precipitation (Kieser, 1984). DNA digestion with restriction enzymes, dephosphorylation with alkaline phosphatase, filling in of cohesive ends with the large fragment of DNA polymerase I (Klenow fragment), and ligation with T4 DNA ligase were performed according to Sambrook *et al.* (1989). DNA restriction fragments were purified by elution from low-melting-point agarose gels as described by Parker & Seed (1980). Transformation of *E. coli* JM109 was carried out according to Chung *et al.* (1989).

Nucleotide sequence analysis. This was carried out in an automated DNA sequencing system (ALF-sequencer; Pharmacia) as described previously (Pelletier *et al.*, 1994), using the fluorescent-labelled universal primer (UP) and reverse primer (RP) and the AutoRead Sequencing Kit from Pharmacia. Double-stranded DNA of pUC19-derivative plasmids used as the template for DNA sequencing was prepared with the Quiagen DNA preparation kit (QIAwell-8 Plasmid Kit). Strong compressions in sequencing gels due to stable secondary structures were resolved by the use of 7-deaza dATP in addition to 7-deaza dGTP provided by the kit. The computer analysis of sequence data was performed on a Microvax work station 3200, using the Sequence Analysis Software Program version 7.3 of the University of Wisconsin, Genetics Computer Group (Devereux *et al.*, 1984). The statistical analysis of codon utilization was performed with the CODON PREFERENCE program (Gribkov *et al.*, 1984), using a codon usage table of *Pseudomonas* generated from the analysis of 259 *Pseudomonas* genes (Wada *et al.*, 1992). Database searches were run with the programs BLASTN, BLASTP and TBLASTN (Altschul *et al.*, 1990) on the BLAST electronic-mail server from the National Center for Biotechnology Information, Bethesda, MD, USA. Protein alignments were performed with the programs BESTFIT, LINEUP and PILEUP.

RESULTS

Subcloning and nucleotide sequence analysis of the esterase gene

The nucleotide sequence of the 1.2 kb *Pst*I fragment on pUE1251 has been newly determined, using a double-

stranded DNA sequencing protocol. The sequencing strategy is shown in Fig. 1 which indicates the deletions generated at either end of the insert to get the overall DNA sequence on both strands, using the fluorescent UP and RP primers. Fig. 2 shows the nucleotide sequence of the esterase gene, corrected at 15 positions in comparison with the one published previously (Choi *et al.*, 1990). Computer analysis of the sequence on both strands allowed the identification of one large ORF, matching perfectly the codon usage of *Pseudomonas* genes (not shown). This 816 bp ORF extends from the start codon ATG at position 248 to the stop codon TGA at position 1064 and encodes a predicted protein of 272 amino acids. The average G + C content (62 mol%) of the esterase encoding sequence is typical for *Pseudomonas* genes.

In contrast, the codon preference analysis of the published esterase gene showed a low accordance with the *Pseudomonas* codon usage, with the presence of a large number of 'rare codons' in the central part of the gene and at its 3' end. It was caused by five frameshifts in the coding sequence of the esterase, generated in regions particularly difficult to sequence.

The 5' end of the esterase gene is in agreement with the one previously described. Furthermore, the N-terminal part of the predicted protein sequence is identical with the one determined by Edman degradation of the purified esterase, which gave the sequence S-T-F-V-A-K-D-G-T-Q-I-Y-F-K-D-W-G (Choi *et al.*, 1990). However, the revised ORF encodes a putative protein of 272 amino acids, with a calculated M_r of 30092, instead of a 236 amino acid protein with a calculated M_r of 26936 as previously published. The new M_r of the deduced protein was more in agreement with the M_r of the esterase estimated by SDS-PAGE (29.5 kDa).

The esterase gene (Fig. 2) is preceded by a putative Shine-Dalgarno motif, AGGAG, 7 bp upstream of the start codon, and potential *E. coli*-type promoter sequences

Fig. 1. Sequencing strategy of the 1.2 kb *Pst*I DNA fragment on pUE1251. Different deletions of the 1.2 kb insert were generated using a restriction site in the polycloning site and a second within the insert, as indicated below each arrow. The arrows indicate the nucleotide sequence obtained for each subclone, using the UP (filled leftwards arrows) and RP (diagonally hatched rightwards arrows).

1 (*Sau3A*)/*AatII*
GATCTGGACGTCTCGGCCCGCGCGCGCGCTCGAAGGTGAAGGGCTTGAGGGCATCCTTGGCGTTCTGGGCGCGCTAGCTGTAAGTC
CTAGACCTGCAGGAGCCGGGGGCGCGCGCGCGCAGCTTCCACTTCCCGAACTCCCGTAGGAACCGCAAGACCCGCGCATCGACATTGAG

91 -35 -10 °----->
TTGGCCATTTCGTTACCTGTGTGATCTGACGGTAGAGGTGAGTGACCATAGACGCTGAACCGTTCAACCGAGCACACCGGTACAA
AACCGGTAAAGCAAGTGGACACACTAGACTGCCATCTCCAGTCACTGGTTCGTATCTGCGACTTGGCAAGTTGGCTCGTGTGGCCATGTT

181 -35 -10 RBS
GCCCTTCGTCGGTTCACGCAATAGTGCCTAGAGTCTTTAGCCCCCTCAACCAGGAGTTTTTGCATGAGCACATTGTTGCAAAAGA
CGGGAAGCAGGCCAAAGTGCCTTATCAGCGATCTCAGAAAGTCGGGGGAGTTGGTCCTCAAAAACGTACTCGTGTAAACAACGTTTTCT
M S T F V A K D 8

271 *KpnI* *BglII*
CGGTACCCAGATCTATTTCAAGGACTGGGGCAGCGGTAAACCGGTGTGTTTACGCCACGGTTGGCTACTGGATGCCGACATGTGGGAATA
GCCATGGGTCTAGATAAAGTTCTGACCCCGTCGCCATTTGGCCACAACAAGTCGGTGCCAACCGATGACCTACGGCTGTACACCCCTTAT
Q T Q I Y F K D W Q S G K P V L F S H G W L L D A D M W E Y 38

361
CCAGATGGAGTACCTCAGCAGCCGCGGTATCGCACCATCGCCTTTGACCGCGCGGCTTTGGCCGCTCGGACCAACCCCTGGACCGGCAA
GGTCTACCTCATGGAGTCGTCGGCGCGGATAGCGTGGTAGCGGAACTGGCGGCGCGGAAACCGCGAGCGCTGGTTGGGACCTGGCCGTT
Q M E Y L S S R G Y R T I A F D R R G F G R S D Q P W T G N 68

451 *NcoI*
CGACTACGACACCTTCGCCGACGACATCGCCAGTTGATCGAACACCTGGACCTCAAGGAGGTGACCTGGTGGGCTTCTCCATGGGCGG
GCTGATGCTGTGGAAGCGGCTGCTGTAGCGGGTCAACTAGCTTGTGGACCTGGAGTTCTCCACTGGGACCAACCGAAGAGGTACCCGCC
D Y D T F A D D I A Q L I E H L D L K E V T L V Q F S M G 98

541 *NaeI*
CGGCGATGTGGCCGCTACATCGCCCGCCACGGCAGCGCACGGGTGGCCGGCTGGTGTCTGGGCGCCGTCACCCCGCTGTTCCGCCA
GCCGCTACACCGGGCGATGTAGCGGGCGGTGCGTTCGCGTGCCACCGGCCGACACGACGACCCGCGGAGTGGGGCGACAAGCCGGT
G D V A R Y I A R H G S A R V A G L V L L G A V T P L F G Q 128

631
GAAGCCGACTATCCGAGGGTGTCCCGTTCGATGTGTTTCGCAAGGTTCAAGACTGAGCTGTGAAGGATCGCGCGAGTTCATCAGCGA
CTTCGGGCTGATAGCGTCCACAGGGCGAGCTACACAAGCGTTCCAAGTTCTGACTCGACGACTTCCTAGCGCGGTCAAGTAGTTCGCT
K P D Y P Q G V P L D V F A R F K T E L L K D R A Q F I S D 158

721 *BglI*
TTTCAACGCACCGTTCTATGGCATCAACAAGGGCCAGGTCGTCTCCCAAGGCGTGACAGCCAGACCCCTGCAAAATCGCCCTGCTGGCCTC
AAAGTTGCGTGGCAAGATACCGTAGTTGTTCCCGGTCCAGCAGAGGGTTCCGCACGTCTGGGTCTGGGACGTTTAGCGGGACGACCGGAG
F N A P F Y G I N K G Q V V S Q G V Q T Q T L Q I A L L A S 188

811
GCTCAAGGCCACGGTGGATTGCGTCAACCGGTTCCGCCGAAACCGACTTCCGCCCGGACATGGCCAAGATCGACGTACCCACCCCTGGTGAT
CGAGTTCCCGGTGCCACCTAACGCAGTGGCGCAAGCGGCTTTGGCTGAAGGCGGGCTGTACCGGTTCTAGCTGCATGGGTGGGACCACTA
L K A T V D C V T A F A E T D F R P D M A K I D V P T L V I 218

901 *NcoI* *BclI*
CCATGGCGATGGCGACAGATCGTCCGTTTCGAGACCAACCGGCAAGTGGCGGCGGAGTTGATCAAGGGCGCGCAACTGAAGGTGTACAA
GGTACCGCTACCGTGGTCTAGCACGGCAAGCTCTGGTGGCCGTTTACC CGCGCTCAACTAGTTCCCGCGGCTTGACTTCCACATGTT
H G D G D Q I V P F E T T G K V A A E L I K G A E L K V Y K 248

991
GGACGCGCCCCACGGTTCGCGGTGACCCACGCCAGCAGTTGAACGAAGACCTGTTGGCGTTCTTGAAACGCTGAGTTAATGTCTGCCT
CCTGCGCGGGTGCCCAAGCGCACTGGGTGCGGTCGTCAACTTGCTTCTGGACAACCGCAAGAACTTTGCGACTCAATTACAGACGGA
D A P H G F A V T H A Q Q L N E D L L A F L K R * 272

1081 *PvuI* °----->
TTATCGGCCGGGCTTGGCCCGGCTTTTTTTTACGCGCTCGAATCTCGCGGGTCGCCGGGCAAGATCGTGAACCGCGCCCTCGC
AATAGCCGGCCCAACGGGCGGAAAAAATGCGCGAGCTTAGAGCGCGCCAGCGGCCGCTGCTAGCACGACTTGGCGGCGGGGAGCG

1171 *PstI*
CACGGTCCCAACCCATACGTTTCATCGCTGCAG
GTGCCAGGGTTGGGTATGCAAGTAGCGACGTC

Fig. 2. For legend see facing page.

Fig. 3. Alignments of the esterase of *P. fluorescens* with the bacterial non-haem haloperoxidases BPO-A1 (Pelletier *et al.*, 1994) and BPO-A2 (Pfeifer *et al.*, 1992) (bromoperoxidases from *S. aureofaciens*), CPO-P (a chloroperoxidase from *P. pyrrocinia*, Wolframm *et al.*, 1993) and CPO-L (a chloroperoxidase from *S. lividans*, Bantleon *et al.*, 1994). Identical residues are indicated by a dark grey background and conservative substitutions by a pale grey background. The amino acids considered as similar are: (D, E), (N, Q), (R, K), (A, S, T), (I, L, M, V) and (F, Y, W). The end of each protein is indicated by an asterisk. The catalytic serine-motif around position 101 is boxed. The aspartate and histidine residues which are thought to be involved in the catalytic triad are marked with a diamond.

(−35 and −10) have also been found in the 5′-flanking region. Furthermore, a perfect inverted repeat is present 19 bp downstream of the TGA termination codon, between positions 1086 and 1103, giving a potential stem-loop structure with a calculated ΔG of $-23 \text{ kcal mol}^{-1}$ (-96 kJ mol^{-1}). The presence of a poly(T) stretch immediately downstream of the palindromic sequence suggests that this structure is a rho-independent transcription terminator of the esterase gene (Rosenberg & Court, 1979).

Homology between the esterase and bacterial non-haem haloperoxidases

Using the BESTFIT and LINEUP programs, the deduced amino acid sequence of the esterase was compared with four bacterial non-haem haloperoxidases with known sequences: BPO-A1 (Pelletier *et al.*, 1994) and BPO-A2

(Pfeifer *et al.*, 1992), the two bromoperoxidases from *S. aureofaciens* ATCC 10762; CPO-P, the chloroperoxidase from *P. pyrrocinia* (Wolframm *et al.*, 1993); and CPO-L, the chloroperoxidase from *S. lividans* (Bantleon *et al.*, 1994). The esterase and haloperoxidases displayed a high degree of similarity in length, from 272 amino acid residues for the esterase to 278 residues for BPO-A2. The overall primary structures of the five proteins were highly related, permitting a multi-alignment (Fig. 3) with only a few gaps to generate an optimal matching of all sequences. Highly conserved domains were found in the N-terminal half of the five proteins, extending over the first 150 amino acids. In the second half, two shorter conserved regions were identified, located between residues 210 and 240, and from position 255 to the C-terminal end. The similarity score of the five proteins along their overall primary structures is plotted in Fig. 4(a). It indicates a more variable domain in the central part of the halo-

Fig. 2. Revised nucleotide sequence of the esterase gene from *P. fluorescens* and its flanking regions, differing at 15 positions compared with the sequence data published by Choi *et al.* (1990). The *Pst*I and *Sau*3A sites used for the cloning of the esterase gene after partial digestion are indicated. The restriction sites used to generate deletions in the esterase-encoding fragment are shown. Numbering on the left side refers to the nucleotide sequence and numbering on the right to the deduced amino acid sequence displayed below with the one-letter code. The N-terminal portion of the esterase previously determined by Edman degradation is indicated in bold letters and underlined. The conserved region surrounding the active-site serine residue Ser95 is double-underlined. The potential −10 and −35 promoter sequences and ribosome-binding site identified in the 5′ non-coding region of the esterase gene are indicated. A 9 bp sequence directly repeated upstream and downstream of the esterase gene is labelled (°). The palindrome sequence followed by a poly(T) stretch in the 3′ flanking region is presumably the transcriptional terminator of the gene.

Fig. 4. Similarity plot and phylogenetic tree of the esterase and the bacterial haloperoxidases. (a) The similarity score between the protein sequences previously aligned with the PILEUP program is calculated along the overall alignment between the amino acid positions 1 and 285 (numbering refers to Fig. 3). The plot above the dashed line indicates the presence of highly conserved domains in the five proteins. (b) Phylogenetic tree of the bacterial non-haem haloperoxidases and the esterase of *P. fluorescens*.

peroxidases and the esterase between positions 150 and 210. Considering the putative heterogeneity of substrate specificity among the bacterial haloperoxidases (Weng *et al.*, 1991; Wolframm *et al.*, 1993), this variable domain should be involved in substrate recognition.

To determine the evolutionary distance between the esterase and haloperoxidases, a phylogenetic tree was constructed by using the PILEUP program. As shown in Fig. 4(b), the four haloperoxidases from bacterial sources are closely related to each other, forming an homogenous enzyme family. The esterase of *P. fluorescens* fits perfectly into the haloperoxidase family, sharing the greatest number of identical amino acids with CPO-L and BPO-A1 (50% and 46% identical amino acids, respectively). However, the highest overall score of similarity was calculated for BPO-A1 (68.8%). Furthermore, we observed that the esterase is even more closely related to the haloperoxidase group containing BPO-A1, CPO-L and CPO-P than is BPO-A2.

Purification of the esterase of *P. fluorescens* produced in *E. coli* JM109(pUE1251)

Protein samples prepared from *E. coli* JM109(pUE1251) grown under inducing conditions were analysed by SDS-PAGE. A protein with an M_r of 29 500 was produced in large amounts by JM109 cells bearing pUE1251 but was absent in extracts from JM109 carrying the plasmid without insert. The clone carrying pUE1251 was tested for its ability to display esterase activity on agar plates overlaid with an emulsion of 1% tributyrin. Esterase activity could be detected by the formation of a halo of hydrolysis, in contrast with the clones carrying pIP50 (encoding CPO-P) or pUC19, which did not produce haloes on this medium (data not shown).

In preliminary experiments, crude extracts of *E. coli* JM109(pUE1251) were loaded onto native PAGE gels and stained either for esterase activity with *p*NPA or for brominating activity with phenol red. We observed that the esterase of *P. fluorescens* exhibits both types of activity. However, attempts to detect the brominating activity of the esterase in the spectrophotometric assays with MCD as substrate were unsuccessful. This apparent discrepancy has been previously reported in crude extracts of *E. coli* (Wolframm *et al.*, 1993) and *S. lividans* (Weng *et al.*, 1991). It is assumed that in crude extracts an inhibitor of bromination is present or a competitive reaction occurs, leading to the depletion of one of the substrates required for bromination. Fractionation of crude extracts on native PAGE avoids this inhibitory effect but purification of crude extract is required prior to performing spectrophotometric measurements.

Crude extract of *E. coli* JM109(pUE1251) (50 mg protein) was fractionated by anion-exchange chromatography. The elution profile displayed a sharp peak of esterase activity eluted with about 0.5 M sodium chloride. This purification step resulted in a 7.4-fold enrichment of the esterase, with a 70% recovery. The specific esterase activity in the standard assay with *p*NPA as substrate was 89 U (mg protein)⁻¹. No comparison can be made with published results which have been obtained with the substrate methyl acetyl salicylate (Choi *et al.*, 1990). The partially purified esterase gave a highly dominant band with an apparent M_r of 29 500 as measured by SDS-PAGE.

The pI of the esterase was determined by isoelectric focusing of the purified esterase in the native form. The esterase stained with Coomassie blue for protein and with *p*NPA for esterase activity migrated with a pI of 4.4, as determined by simultaneous migration of calibration

proteins. This value is in good agreement with the calculated isoelectric point of the esterase subunit ($pI = 5.4$), in contrast with the pI of 11.9 deduced from the previously published esterase sequence. The pI of 4.4 is also compatible with experimental observations: the esterase was purified by anion-exchange chromatography with a buffer system at pH 7, indicating a pI below 7.

To carry out comparative studies of the esterase and CPO-P, it was necessary to purify CPO-P from crude extract of *E. coli*. Fractionation of crude extract by anion-exchange chromatography was performed using the same procedure as that used for the esterase. The brominating activity of CPO-P was enriched 7.5-fold, with a 74 % recovery. The specific brominating activity of CPO-P was 47 U (mg protein)⁻¹. The published value (Wolfframm *et al.*, 1993) for the purified enzyme is 63 U (mg protein)⁻¹, indicating that we obtained CPO-P with a degree of purification of 75 %.

In situ detection of brominating and esterase activities

Fractions enriched in the esterase and CPO-P obtained by anion-exchange chromatography were loaded onto native PAGE gels and stained either for brominating activity or for esterase activity (data not shown). Whereas, *in situ* CPO-P exhibited only brominating activity, the esterase of *P. fluorescens* showed both activities. However, 20 µg protein was required to detect the brominating activity in the case of the esterase, whereas only 0.05 µg protein was necessary for CPO-P. These results indicated firstly, that the esterase is a bifunctional enzyme, and secondly, that the esterase is not only structurally, but also functionally related to haloperoxidases. Furthermore, the haloperoxidases BPO-A1, BPO-A2 and CPO-L, tested in the same way by native PAGE, showed the same behaviour as CPO-P: they exhibited only brominating activity. Esterase activity could not be detected *in situ* when phenyl acetate, 2-naphthyl acetate or tributyrin were used as substrates (data not shown).

Catalytic features of the esterase and of CPO-P

The esterase activity of the purified esterase was measured spectrophotometrically using pNPA as substrate. The pH optimum for esterase activity in potassium phosphate buffer was above 9. Maximum esterase activity was observed at 70 °C. An apparent K_m for pNPA of 92 µM was estimated from Lineweaver-Burk plots. The purified fraction of CPO-P was also assayed for esterase activity by the colorimetric assay with pNPA. Whereas CPO-P did not exhibit any detectable esterase activity at pH 5.5, hydrolysis of pNPA could be measured at higher pH. A maximum specific esterase activity of 1.8 U (mg protein)⁻¹ was obtained at pH 8.5 for CPO-P.

The brominating activity of the esterase was also studied spectrophotometrically. The specific brominating activity of the esterase was 0.1 U (mg protein)⁻¹, using the MCD assay. For comparison, the specific brominating activity of purified CPO-P was 47 U (mg protein)⁻¹. The two

brominating activities could be differentiated by their pH and temperature optima. Maximum brominating activity in 1 M sodium acetate was observed at pH 6 and 55 °C for CPO-P, but at pH 5 and 50 °C for the esterase.

DISCUSSION

Screening GenBank with the TBLASTN program indicated that an esterase from *Pseudomonas fluorescens* had significant homology with the bacterial haloperoxidases. However, this homology was only found in two distinct domains of the published esterase sequence. As errors in the published sequence were suspected, the esterase gene was sequenced again (Figs 1 and 2).

The deduced amino acid sequence differs from the one published due to the correction of five frameshifts. When scanned with the CODON PREFERENCE program, the corrected ORF encoding the esterase is now in very good agreement with the codon usage of *Pseudomonas* genes. The predicted esterase sequence displays a very high score of identical amino acids with all haloperoxidases along their overall sequences (Fig. 3), between 40 % with BPO-A2 and 50 % with CPO-L. However, the similarity plot (Fig. 4a) displays more strongly conserved domains in the N-terminal and at the C-terminal ends of the five proteins.

The relationship of the esterase to the bacterial haloperoxidase family can be visualized by a phylogenetic tree (Fig. 4b). This points to a probable common ancestor for the esterase, BPO-A1, CPO-L and CPO-P, whereas an earlier divergence could have occurred for the BPO-A2 ancestor. Immunological studies of the esterase have been carried out to check for a putative cross-reaction between the esterase and haloperoxidases. The esterase was detected on Western blots using antibodies raised against CPO-P (kindly provided by K.-H. van Pée), confirming their structural relationship (unpublished results).

Biochemical investigations were performed to determine whether the structural homology between the esterase and haloperoxidases also reflected functional homology. Preliminary experiments revealed that the esterase, expressed in *E. coli* cells containing pUE1251, displayed in addition to the expected esterase activity, the characteristic staining pattern for brominating activity in the phenol red assay used routinely for haloperoxidases. However, attempts to measure brominating activity in the spectrophotometric assay with MCD as substrate were only successful after fractionation of crude extracts of *E. coli* containing pUE1251 by anion-exchange chromatography. In the purification protocol reported by Choi *et al.* (1990), the esterase was isolated from the periplasmic fraction of *E. coli* cells, despite the lack of a potential signal peptide in the predicted protein sequence. Several attempts to isolate the esterase from *E. coli* after an osmotic shock, performed essentially as described by LaVallie *et al.* (1993), were unsuccessful. The esterase was always predominantly found in the cytoplasmic fraction of cells and less than 2 % of total esterase activity was recovered in the supernatant after this treatment. Therefore, crude extracts of total cells had to be prepared. Fractionation of cell extracts containing CPO-P and the

esterase by anion-exchange chromatography resulted in an enrichment of 7.5-fold for both enzymes, thus permitting comparative studies.

The enriched fraction of the esterase showed a specific brominating activity of 100 mU (mg protein)⁻¹ in the MCD assay, whereas the purified fraction of CPO-P showed 47 U (mg protein)⁻¹. This indicates that the esterase of *P. fluorescens* is able to perform the bromination of MCD, but at a lower efficiency than CPO-P. The enriched fraction of the esterase displayed a specific esterase activity of 89 U (mg protein)⁻¹ for the hydrolysis of *p*NPA at pH 7. With CPO-P, no detectable esterase activity was observed *in situ* but significant esterase activity was detected in the colorimetric assay with *p*NPA. The enriched fraction of CPO-P gave a specific esterase activity of 1.8 U (mg protein)⁻¹ at pH 8.5. Furthermore, significant esterase activity was also measured spectrophotometrically in crude extracts of *S. lividans* overexpressing BPO-A1, BPO-A2 or CPO-L (unpublished results). This shows that the bacterial haloperoxidases are also capable of performing the hydrolysis of *p*NPA, but in this case at a lower efficiency than the esterase of *P. fluorescens*.

The esterase of *P. fluorescens* is the first known example of another type of enzyme related to the bacterial non-haem haloperoxidases, sharing with them common features at the structural, immunological and catalytic levels. The fact that the esterase displays brominating activity indicates that it possesses the entire catalytic machinery required to perform the oxidation of Br⁻ during halogenation. These findings are important because the catalytic mechanisms of halogenation by the bacterial haloperoxidases is very poorly understood. Haag *et al.* (1991) postulated that a methionine residue could be involved in the catalytic process. However, counter-evidence was obtained when more bacterial haloperoxidase genes were sequenced since no methionine was found conserved at a similar position in six different bacterial haloperoxidases. Consequently, it seems unlikely that one of these non-conserved residues plays a crucial role in the catalytic mechanism of halogenation.

Esterases belong to the large enzyme family of serine-hydrolases, comprising various proteases, lipases, esterases and other hydrolases. They are characterized by the presence of a catalytic triad in their active site (Brady *et al.*, 1990; Schrag *et al.*, 1991) responsible for the hydrolysis of ester or amide bonds, which includes a nucleophilic amino acid, usually serine, an acidic residue (aspartic or glutamic acid) and finally a conserved histidine residue. Noteworthy is the conservation of a consensus motif GlyX₁-Ser-X₂-Gly, around the nucleophilic active serine residue, due to potential steric constraints (Ollis *et al.*, 1992). Of special interest was the identification of the motif Gly-Phe-Ser-Met-Gly in the esterase sequence, around Ser95 as indicated in Fig. 2, matching perfectly the consensus pattern. This result differs from the previously reported data in which no serine motif could be identified in the esterase sequence (Choi *et al.*, 1990; Kim *et al.*, 1993). The presence of the serine consensus motif in the

esterase confirmed that this enzyme is a typical serine hydrolase which performs hydrolysis of ester bonds after nucleophilic attack of the hydroxyl group from the active serine residue. Studies of inhibition by PMSF, a serine-hydrolase inhibitor (Gold & Fahrney, 1964; Kraut, 1977), further support the involvement of the active serine in the catalytic mechanism of the esterase (unpublished results).

Very surprisingly however, the serine-consensus motif was present in all haloperoxidases, at the same position as in the esterase sequence, in a strongly conserved domain around position 101 in the multi-alignment (Fig. 3). The presence of the characteristic motif of serine-hydrolases in the bacterial non-haem haloperoxidases strongly emphasizes the structural and functional relationship between both enzyme families, with the esterase of *P. fluorescens* as a link between them.

Despite a quite low overall sequence homology, it was possible to align the haloperoxidases and esterase sequences with various serine-hydrolases such as the diene lactone hydrolase, DLH, from *Pseudomonas* sp. B13 (Frantz *et al.*, 1987) and the dehalogenase, Hal, from *Xanthobacter autotrophicus* (Janssen *et al.*, 1989), (data not shown). The three-dimensional structure of DLH and Hal has been elucidated by X-ray diffraction (Pathak & Ollis, 1990; Franken *et al.*, 1991; Verschueren *et al.*, 1993). Ollis *et al.* (1992) have shown that they belong to the structural family of serine-hydrolases characterized by the α/β hydrolase fold. When we superimposed the elements of secondary structure of Hal and DLH in the sequence multi-alignment with haloperoxidases and the esterase, we observed a striking conservation of amino acids in regions forming the β -strands in Hal and DLH, which constituted the backbone of the α/β hydrolase fold (unpublished results). These structural homologies led us to assume that the haloperoxidases and esterase share a similar three-dimensional organization, with the conservation of the hydrolase fold structure. Elucidation of the three-dimensional structure of BPO-A2 is under way and should confirm this hypothesis.

The above structural model allowed us to identify putative candidates for the acidic and histidine residues aligned with those of Hal and DLH, which could form, together with the previously described conserved serine, a catalytic triad in the haloperoxidases and esterase. The catalytic triad of the esterase of *P. fluorescens* presumably comprises the residues Ser95, Asp223 and His252 (numbering refers to Fig. 2). A catalytic triad structurally equivalent to the one found in the esterase, Hal and DLH, is also present in haloperoxidase sequences (Fig. 3), in highly conserved domains. This triad is very likely to be involved in the catalytic process leading to the hydrolysis of *p*NPA displayed by the haloperoxidases. It is important to determine whether this triad also plays a role in the catalytic process involved in halogenation. Studies of the inhibition of CPO-P, BPO-A1 and BPO-A2 by PMSF indicate that the serine in the conserved motif plays a crucial role in halogenation (unpublished results). Site-directed mutagenesis experiments are in progress, in order to exchange the putative active residues Ser97,

Asp229 and His258 in CPO-P, analogous to the triad of the esterase, and to confirm the involvement of a functionally active catalytic triad in halogenation (I. Pelletier, unpublished). However, based on the high sequence similarities with the esterase, on the predicted structural homologies with various serine-hydrolases containing the α/β hydrolase fold structure and on the presence of a structurally similar catalytic triad, we conclude that the bacterial non-haem haloperoxidases belong to the serine-hydrolase family.

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