Protein engineering of the 2-haloacid halidohydrolase IVa from *Pseudomonas cepacia* MBA4

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The chemical modification of L-2-haloacid halidohydrolase IVa (Hdl IVa), originally identified in *Pseudomonas cepacia* MBA4, produced as a recombinant protein in *Escherichia coli* DH5 α , led to the identification of histidine and arginine as amino acid residues likely to play a part in the catalytic mechanism of the enzyme. These results, together with DNA sequence and analyses [Murdiyatmo, Asmara, Baines, Bull and Hardman (1992) *Biochem. J.* **284**, 87–93] provided the basis for the rational design of a series of random- and site-directed-mutagenesis experiments of the Hdl IVa structural gene (*hdl* IVa). Subsequent apparent kinetic analyses of purified mutant enzymes identified His-20 and

Arg-42 as the key residues in the activity of this halidohydrolase. It is also proposed that Asp-18 is implicated in the functioning of the enzyme, possibly by positioning the correct tautomer of His-20 for catalysis in the enzyme–substrate complex and stabilizing the protonated form of His-20 in the transition-state complex. Comparison of conserved amino acid sequences between the Hdl IVa and other halidohydrolases suggests that L-2-haloacid halidohydrolases contain conserved amino acid sequences that are not found in halidohydrolases active towards both D- and L-2-monochloropropionate.

INTRODUCTION

Haloacid halidohydrolases (Goldman et al., 1968) are hydrolytic dehalogenases which cleave the carbon–halogen bond(s) in 2-haloalkanoic acids, generating hydroxyacids from mono-substituted compounds and oxoalkanoic acids from disubstituted substrates. Generalized mechanisms for halidohydrolase activity have been proposed (Little and Williams, 1971; Weightman et al., 1982). These mechanisms predict that the enzyme catalyses hydrolysis of the carbon–halogen bond by the nucleophilic attack of cysteine or histidine residues. The proposed mechanism also requires the presence of a positively charged amino acid, namely lysine or arginine, to co-ordinate with the acid carboxy group of the substrate.

Pseudomonas cepacia MBA4 (Tsang et al., 1988b) expresses only a single 2-haloacid halidohydrolase in batch culture, designated halidohydrolase IVa (Hdl IVa). The structural gene for Hdl IVa (*hdl* IVa) has been cloned (Tsang et al., 1988a), and sequenced (Murdiyatmo et al., 1992) and shown to encode a 231-amino-acid-residue protein (M_r 25900). Whilst acknowledging the uncertainty associated with all secondary-structure predictions made on the basis of a primary nucleotide sequence, data obtained from such analyses of the *hdl* IVa sequence provided the basis for an interesting preliminary model. The basic principles of this model have been supported by biochemical studies of this enzyme, which revealed that its activity was insensitive to thiol-group-reactive reagents and that it demonstrated chiral specificity towards L-2-monochloropropionic acid (2MCPA; Tsang et al., 1988b). In order to investigate the model further, chemical modification of the Hdl IVa protein and *in vitro*, random and site-directed mutagenesis of the recombinant *hdl* IVa contained on plasmid pUKC510 (Murdiyatmo et al., 1992) was undertaken in order to identify the region of the amino acid sequence which encoded the active site of this halidohydrolase.

MATERIALS AND METHODS

Culture and maintenance conditions

The recombinant *Escherichia coli* strains used in the present study were cultured in NaCl-free L-broth (1% tryptone/0.5% yeast extract), supplemented with ampicillin (50 mg·ml⁻¹) where appropriate. L-broth containing 2MCPA and Bromothymol Blue, as described by Kawasaki et al. (1984), was used to select the mutated recombinant strains.

Measurement of halidohydrolase activity

The preparation of cell-free extract (CFE) and the measurement of halidohydrolase activity were made as previously described (Hardman and Slater, 1981). Halide ion release was normally measured using a Marius Chlor-o-counter (F. T. Scientific Instruments Ltd., Tewkesbury, Glos., U.K.). The halidohydrolase assays, for kinetic studies were performed in triplicate at 30 °C in 10 mM Tris/H₂SO₄ buffer, pH 8.0, with 70–300 μ M monochloroacetic acid (MCA) or 2MCPA as the substrate. Cl⁻ released in the assay mixture was measured by the colorimetric method of Bergmann and Sanik (1957) using a Lambda 15UV/VIS spectrophotometer (Perkin–Elmer). Samples (1.0 ml) were taken from the assay mixture at timed intervals and 100 μ l of Reagent A [0.25 M FeNH₄(SO₄)₂·12H₂O in 9 M HNO₃] and the whole mixed thoroughly. Aliquots (1.0 ml) were then transferred to a cuvette, mixed with 91 μ l of Reagent B [95% ethanol saturated with Hg(SCN)₂], and the absorbance measured at 460 nm. Cl⁻ was then determined by reference to a standard curve constructed with 0–0.2 μ mol of Cl⁻. The ENZPACK program (Elsevier–Biosoft, Cambridge, U.K.) was used to analyse the enzyme-kinetic data.

PAGE

An AgNO₃ activity stain (Tsang et al., 1988b) was used to reveal

Abbreviations used: Hdl IVa, L-2-haloacid halidohydrolase; *hdl* IVa, its structural gene; 2MCPA, 2-monochloropropionic acid; CFE, cell-free extract; MCA, monochloroacetic acid; DEP, diethyl pyrocarbonate; PG, phenylglyoxal hydrate; DSP, dithiobis(succinimidyl propionate); NEM, N-ethylmaleimide.

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the halidohydrolase activity after PAGE of CFEs and purified proteins.

Purification of halidohydrolase

The purification of halidohydrolase protein from cell free extracts of recombinant *E. coli* strains was achieved using the method described by Tsang et al. (1988b).

Immunoblot analysis

Immunoblot analysis of purified enzyme on SDS/PAGE was also used to confirm the identity of the mutant and the wild-type halidohydrolases. Proteins were transferred from the gel to a nitrocellulose sheet by electrophoresis (Towbin et al., 1979). Immuno-cross-reactivity was detected by the method of Leary et al. (1983), using antiserum raised against Hdl IVa purified from *Pseudomonas cepacia* MBA4 and a goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate.

Amino acid modification

Modification of histidine residues was achieved using a range of concentrations of diethyl pyrocarbonate [DEP; 0.5–2.0 mM in 30 % (v/v) ethanol] for 2–10 min (Meyer and Cromartie, 1980; Tsurushii et al., 1975). Samples of the purified enzyme preparations (110 µg in 190 µl of 100 mM sodium borate buffer, pH 7.0) were treated with 10 µl of DEP solution and incubated at 25 °C. At the appropriate time intervals the reaction was stopped by the addition of 100 mM Tris/H₂SO₄, pH 8.0 (5000 µl) and the enzyme preparation assayed for activity. The reaction mixture for modification of arginine residues contained 10 µl of phenylglyoxal hydrate (PG; 1.0–100 mM) dissolved in 100 mM sodium borate buffer, pH 8.0, and the reaction was carried out according to the procedures described by Takahashi (1968). Cysteine residues were modified by incubating enzyme preparations in various concentrations of *N*-ethylmaleimide (NEM; 1.0–5.0 mM) in the assay buffer for 10 min at 25 °C. The remaining enzyme activity was then assayed, the enzyme reaction being started by the addition of MCA as the substrate. Lysine-residue modification was achieved by adding dithiobis-succinimidyl propionate (DSP; 1.0–10 mM) to the enzyme preparations (Lomant and Fairbanks, 1976). In instances where the chemical modification was shown to affect activity, experiments were repeated in the presence of MCA (0.5 M), a substrate for the enzyme, to examine whether the presence of substrate-protected residues in the active site from modification.

Preparation of plasmid DNA

The method of Johnson (1990) was used for rapid small-scale preparations, whilst large-scale preparation was achieved using the Brij-lysis method (Clewett and Helsinki, 1969), followed by caesium chloride/ethidium bromide buoyant-density centrifugation.

Random *in vitro* mutagenesis of *hdi* IVa

Hydroxylamine was used to introduce random mutations into the recombinant *hdi* IVa carried on pUKC510 (Murdiyatmo et al., 1992) using the method of Rose and Fink (1987). After the treatment with hydroxylamine the plasmid was subjected to *EcoRI*/*HindIII* restriction and the putative mutagenized fragments purified then ligated to the non-mutagenized vector moiety of pUKC510 which had also been digested with *EcoRI*/*HindIII*.

Table 1 Oligonucleotides for PCR primers

Mutation	Sequence
From His-20 to Leu-20	5' TGCTTGACGTTCTTTCCGCCGTGAT 3' 5' ATCACGGCGGAAAGAAGCTCAAGCA 3'
From Arg-42 to Ser-42	5' TCGATGTTGTGGAGTCAACGGCAGC 3' 5' GCTGCCGTTCACTCCACAACATCGA 3'
From His-56 to Leu-56	5' ACACTGATGCTTCAGTATGCG 3' 5' CGCATACTGAAGCATCAGTGT 3'

The recombinant plasmids were then transformed into *E. coli* DH5α. Mutant transformants were selected on L-agar supplemented with 2MCPA, Bromothymol Blue and ampicillin (Kawasaki et al., 1984).

Site-directed mutagenesis of *hdi* IVa

Site-directed mutagenesis was achieved by using a recombinant PCR with overlap extension (Ho et al., 1989; Higuchi, 1990). The *hdi* IVa from pUKC510 was subcloned as an *EcoRI*–*HindIII* fragment into pBluescript SK +/– (Stratagene) to generate a plasmid designated as pUKC520, which was then used as the template DNA for recombinant PCR. The recombinant PCR products were digested with *EcoRI*/*PstI*, purified, and ligated with *EcoRI*/*PstI*-digested pUC9 (Viera and Messing, 1982) and then transformed into *E. coli* DH5α.

Synthesis of oligonucleotides for use as PCR primers

Synthetic oligonucleotides were synthesised by the phosphoramidite method using a model-81A Applied Biosystems synthesizer. The sequences of the mutagenic oligomers are shown in Table 1.

Nucleotide sequencing

Sequencing reactions were performed using the dideoxynucleotide chain-termination method of Sanger et al. (1977). As PCR was used in the site-directed mutagenesis experiments, the whole sequence of the mutated genes was determined to ensure that all mutations derived from these procedures were characterized in terms of the desired and spurious mutations. DNA sequences were analysed with the UWGCG (University of Wisconsin Genetics Computer Group) package through the SERC SEQNET facility.

RESULTS

Amino acid modification of the purified Hdl IVa

The results obtained from the amino-acid-modification studies are summarized in Table 2. Treatment of Hdl IVa with DEP (for the modification of histidine) led to a rapid loss of enzyme activity in the absence of MCA, although the enzyme activity was protected when the enzyme was saturated with substrate prior to treatment with the modifying agent. Similar inhibition and substrate protection was observed when PG was used to modify the arginine residues. Modification of cysteine and lysine residues had little effect on the activity of the enzyme. These results suggested that two of the residues likely to play a part in the catalytic mechanism of the enzyme were histidine and arginine.

Table 2 Effect of specific amino acid modification on the activity of purified halidohydrolase IVa

Results are expressed as percentage activity remaining after treatment, with the activity of untreated enzyme representing the 100% value.

Amino acid	Reagent	Concn. (mM)	Activity remaining (%)	
			With substrate protection*	Without substrate protection
Cysteine	NEM	1.00	ND†	95.58 ± 0.90
		3.00	ND	86.23 ± 1.08
		5.00	ND	83.92 ± 1.38
Histidine	DEP	0.50	70.28 ± 1.58	37.75 ± 0.85
		1.00	65.30 ± 1.07	23.67 ± 1.04
		1.50	62.93 ± 1.31	8.10 ± 1.31
		2.00	50.59 ± 2.72	2.46 ± 0.73
Arginine	PG	60.0	89.30 ± 1.08	47.91 ± 2.91
		80.0	80.77 ± 0.91	16.55 ± 0.92
		100.0	72.87 ± 2.40	3.64 ± 1.11
Lysine	DSP	1.0	ND	96.53 ± 0.75
		5.0	ND	92.38 ± 1.57
		10.0	ND	88.83 ± 0.90

* MCA (0.5 M).

† Abbreviation: ND, not determined.

Table 3 Mutants generated by random mutagenesis

Strain	Amino acid mutated		Specific activity toward MCA*	Activity compared with wt (%)	Electrophoretic mobility†
	From	To			
UKC510/wt	—	—	98.07 ± 3.08	100.00	Hdl IVa
UKC5701	Gly-14	Ala-14	0.42 ± 0.04	0.43	Hdl IV
	Arg-42	Cys-42			
UKC5103	Glu-67	Lys-67	78.19 ± 5.18	79.70	Hdl V
UKC5104	Thr-15	Pro-15	78.24 ± 4.23	79.80	Hdl IVa

* Specific activity (μ mol of substrate converted/min per mg of protein).

† As defined by activity-stain PAGE.

Table 4 Mutants generated by site-directed mutagenesis

Strain	Amino acid mutated		Mutation code	Specific activity toward MCA*	Electrophoretic mobility†
	From	To			
UKC510:R42S	Arg-42	Ser-42	R42S	0.45 ± 0.01	Hdl IV
UKC510:H20L	His-20	Leu-20	H20L	8.34 ± 0.31	Hdl IVa
UKC510:H56L	His-56	Leu-56	H56L	78.39 ± 0.82	Hdl IVa
UKC510:H20L:H56L	His-20	Leu-20	H20L	0.0003 ± 0.0006	Hdl IVa
	His-56	Leu-56	H56L		
UKC510:H20L:M24I	His-20	Leu-20	H20L	6.54 ± 0.39	Hdl IVa
	Met-24	Ile-24	M24I		

* Specific activity (μ mol of substrate converted/min per μ g of protein).

† As defined by activity-stain PAGE.

Random mutagenesis

A library of *hdl* IVa mutants were generated as described in the Materials and methods section, and those which showed a decrease or no catalytic activity were selected and isolated for further study. Three mutants were selected and designated as UKC5101, UKC5103 and UKC5104. CFEs of each mutant were prepared, and the halidohydrolase activities of each compared (Table 3). UKC5101 and UKC5103 expressed halidohydrolases which differed from the wild-type enzyme in terms of their electrophoretic mobility; in Table 3 they are shown as having mobilities equivalent to wild-type halidohydrolases IV and V respectively.

The purified halidohydrolase prepared from CFEs of mutant UKC5104 demonstrated a specific activity towards MCA which was 79.8% that of extracts prepared from the wild-type strain, UKC510 (Table 3). The electrophoretic mobility of the mutant enzyme was the same as that of the wild-type. Comparison of the amino acid sequence of the UKC5104 halidohydrolase, predicted from nucleotide sequence data, with that predicted for the UKC510 enzyme, indicated that the reduced activity again was the result of a single mutation resulting in the conversion of amino acid 15 from threonine into proline. The mutation also led to a slight increase in the K_m for MCA and a concomitant decrease in the MCA specificity constant (K_{cat}/K_m) of the mutant enzyme over that of the wild-type halidohydrolase (Table 5 below). The specific activity of purified enzyme from the UKC5103 was approx. 80% of its wild-type counterpart. However, as noted above, the mutated halidohydrolase demonstrated a different electrophoretic mobility. Nucleotide sequence analysis of the UKC5103 *hdl* IVa led again to the identification of a single amino acid substitution, in that amino acid 67 was converted from glutamic acid into lysine (Table 3). The mutation from an acidic to a basic amino acid also led to an alteration in the enzyme pI value of from 5.10 to 6.0. The UKC5103 halidohydrolase demonstrated a K_m for MCA almost identical

with that of the wild-type enzyme, whilst a slightly reduced K_{cat} led to a specificity constant (K_{cat}/K_m) slightly greater than the UKC5104 halidohydrolase (Table 5 below).

The purified enzyme from the UKC5101 demonstrated a very low specific activity towards MCA, and the enzyme had a slightly different electrophoretic mobility, classified as Hdl IV (Table 3). A study of the catalytic properties of the UKC5101 halidohydrolase showed a considerable increase in K_m and increase in K_{cat} for MCA and, as a consequence, the enzyme had a specificity constant far below that of the wild-type enzyme (Table 5 below). Nucleotide-sequence analysis revealed that two point mutations in *hdl* IVa led to the production of the UKC5101 halidohydrolase. The mutations led to amino acids 14 and 42 being converted from glycine into alanine and from arginine into cysteine respectively (Table 3). Further work would be required to determine which of the mutations led to the observed changes in enzyme activity.

Site-directed mutagenesis

The results from the amino acid modification studies, with supporting data from random mutagenesis experiments, predict that histidine and arginine residues play a part in the catalytic activity of the enzyme. To further assess this prediction His-20 and Arg-42 were targeted for site-directed mutagenesis. The nomenclature used to identify each mutation is defined in Table 4. Characterization by apparent kinetic analyses of mutant and wild-type halidohydrolases was performed using MCA and 2MCPA as substrates. Results of these kinetic experiments are shown in Tables 5 and 6. The UKC510:H20L mutant demonstrated markedly reduced activity; however, there was still a residual

Table 5 Apparent kinetic parameters for hydrolysis of MCA by wild-type and mutant enzymes determined under standard conditions

Source of purified enzyme	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
UKC510	111.90 ± 2.57	122.87 ± 5.22	0.910 ± 0.020
UKC5101	0.361 ± 0.008	202.24 ± 5.42	$(0.184 \pm 0.010) \times 10^{-2}$
UKC5103	79.29 ± 3.71	114.74 ± 4.20	0.651 ± 0.04
UKC5104	79.04 ± 2.66	150.45 ± 5.72	0.525 ± 0.002
UKC510:R42S	0.375 ± 0.002	223.36 ± 2.94	$(0.1168 \pm 0.002) \times 10^{-2}$
UKC510:H20L	9.71 ± 0.64	114.46 ± 4.87	0.085 ± 0.002
UKC510:H56L	84.59 ± 8.42	145.89 ± 16.44	0.580 ± 0.0016
UKC510:H20L:H56L	ND*	ND	ND
UKC510:H20L:M24I	7.21 ± 0.06	119.81 ± 2.45	0.068 ± 0.010

* Abbreviation: ND, not detected.

Table 7 Increase in activation energy of substrate hydrolysis of mutant enzymes over those for the wild-type enzyme (assay conditions: pH 7.9 and 30 °C)

$\Delta(\Delta G)$ ($kJ \cdot mol^{-1}$)	Substrate ...	MCA	2MCPA
5101		15.63	18.29
5103		0.69	-0.58
5104		1.39	2.23
510:R42S		15.86	18.01
510:H20L		5.97	6.66
510:H56L		1.13	1.21
510:H20L:H56L		ND*	ND
510:H20L:M29I		6.54	6.97

* Abbreviation used: ND, not detected.

activity which was 20-fold higher than the UKC510:R42S mutant. This led to a consideration of the second His residue which was present in the N-terminal region of the peptide. The His-56 residue was mutated, and the halidohydrolase activity of the resultant mutant, UKC510:H56L, was reduced by 22% compared with the wild-type enzyme. However, when the two His mutations were combined (UKC510:H20L:H56L), there was effectively a complete loss of halidohydrolase function. The apparent kinetic parameters for this mutated peptide could not be measured, since the reaction rates were below detectable levels ($K_{cat} < 0.0008 s^{-1}$ for MCA and $K_{cat} < 0.0001 s^{-1}$ for 2MCPA).

The K_{cat} value for the UKC510:R42S enzyme (MCA and 2MCPA) was almost 300-fold less than that of wild-type enzyme, whereas the K_m values for both substrates were, respectively, 2- and 5-fold greater than that of the wild-type enzyme. Compared with the wild type, Hdl IVa, the UKC510:H20L mutant showed a 12–14-fold decrease in K_{cat} for MCA and 2MCPA, whilst the K_m values were almost the same as that of the wild-type. UKC510:H56L showed only a slight decrease in its catalytic-centre activity with both substrates, and no significant difference in the K_m values were observed. During the site-directed mutagenesis, a spurious result led to the isolation of the mutant designated as UKC510:H20L:M24I. The mutation led to the substitution of His-20 with Leu as planned, but also Met-24 was substituted with Ile. The apparent kinetic parameters of this mutant are also shown in Tables 5 and 6.

The effect of the mutation on the binding strength of the substrate transition-state complex is reflected in the increase in

activation energy for substrate hydrolysis, as calculated from values of K_{cat}/K_m for mutant (mut) and wild-type (wt) enzymes:

$$\Delta(\Delta G) = -RT \ln[(K_{cat}/K_m)_{mut}/(K_{cat}/K_m)_{wt}]$$

(Wilkinson et al., 1983; Chen et al., 1987; Fersht, 1988; Sierks et al., 1990). The effect of the mutations described above on the activation energies of the resultant halidohydrolases is shown in Table 7.

Immunoblot analysis of purified enzyme on SDS/PAGE showed that all the mutant enzymes possessed a high level of immunoreactivity towards the antibody raised against the wild-type enzyme.

DISCUSSION

Generalized mechanisms for halidohydrolase activity (Little and Williams, 1971; Weightman et al., 1982) predict that these hydrolytic dehalogenases catalyse the cleavage of the carbon-halogen bond by nucleophilic attack of cysteine or histidine residues. Four of each of these amino acids are present in the predicted sequence of Hdl IVa (Murdiyatmo et al., 1992). The amino-acid-modification studies reported here support our previous findings (Tsang et al., 1988b) that Hdl IVa activity is relatively insensitive to inhibition by NEM, indicating that histidine rather than cysteine residues play an important role in the enzyme's activity, such that one or more histidine residues situated at or near the active site of the enzyme are critical for activity of the enzyme. Three of the four histidine residues are present in the first 80 residues. Since the proposed reaction

Table 6 Apparent kinetic parameters for hydrolysis of 2MCPA by wild-type and mutant enzymes determined under standard conditions

Source of purified enzyme	K_{cat} (s^{-1})	K_m (μM)	K_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
UKC510	22.81 ± 0.52	548.51 ± 30.67	0.041 ± 0.001
UKC5101	0.085 ± 0.0042	875.29 ± 180.63	$(0.286 \pm 0.014) \times 10^{-4}$
UKC5103	12.60 ± 0.40	240.25 ± 12.64	0.053 ± 0.002
UKC5104	9.53 ± 0.29	550.91 ± 19.15	0.017 ± 0.001
UKC510:R42S	0.086 ± 0.0012	616.13 ± 169.91	$(0.33 \pm 0.024) \times 10^{-4}$
UKC510:H20L	1.61 ± 0.04	536.03 ± 12.06	$(0.30 \pm 0.003) \times 10^{-2}$
UKC510:H56L	14.40 ± 0.32	548.66 ± 27.45	0.026 ± 0.001
UKC510:H20L:H56L	ND*	ND	ND
UKC510:H20L:M24I	1.47 ± 0.07	558.86 ± 12.52	$(0.264 \pm 0.008) \times 10^{-2}$

* Abbreviation used: ND, not detected.

scheme suggests that the acid carboxy group of the substrate is co-ordinated with an Arg or Lys residue, it is conceivable that all the residues critical to the activity of Hdl IVa are contained with the N-terminal 80 or 90 amino acids, as ten basic residues (Arg and Lys) are also present in this region. The modification studies reported here indicate that arginine, but not lysine, residues are required for halidohydrolase activity.

The data obtained from secondary-structure predictions (Murdiyatmo et al., 1992) suggested that N-terminal residues 1–130 form a highly structured domain containing the active site, whilst the remainder form a relatively less ordered domain, possibly involved in the interactions between individual subunits of the dimeric protein. Such results could not be considered as conclusive, but they provided a rational basis for the application of site-directed mutagenesis to further elucidate the structure–function relationships of Hdl IVa.

The series of random- and site-directed-mutagenesis experiments of the Hdl IVa structural gene and subsequent kinetic analysis of putative mutant enzymes after purification has thus led to a more detailed understanding of this halidohydrolase. It should be noted, however, that verification of the apparent kinetic data awaits a study of the effect of pH on the catalytic activity and active-site pK_a determinations on the mutant enzymes. The choice of amino acid modifications in the site-directed mutagenesis experiments was on the basis that, for His to Leu, the change would have resulted in the insertion of a residue that could not be ionized, and the Arg-to-Ser change was on the basis that they are both hydrophilic amino acids; however, whilst Arg is polar and positively charged, Ser is polar but uncharged. These characteristics of the wild-type amino acids are considered important in determining the activity of the halidohydrolase.

The mutations in the halidohydrolase produced in UKC5101 (Gly-14 and Arg-42) decreased its catalytic-centre activity 180- and 270-fold toward MCA and 2MCPA respectively. The K_m of this mutant enzyme toward MCA and 2MCPA was increased up to 5-fold over the wild-type. The mutant UKC510:R42S produced by site-directed mutagenesis showed similar changes to its apparent kinetics, indicating that Arg-42, not Gly-14, was the residue important in affecting enzyme activity.

The effect on the binding strength of the substrate transition-state complex is reflected by an increase in the activation energy for substrate hydrolysis. The activation energy for MCA and 2MCPA hydrolysis by UKC5101 and UKC510:R42S, when compared with the wild-type enzyme (Table 7), are typical values for the energy loss caused by the elimination of charged groups involved in hydrogen-bond formation with the substrate (Fersht et al., 1985; Sierks et al., 1990). This energy loss was not apparent for UKC5103 or UKC5104 mutants, and these mutants still maintained the wild-type level of catalytic activity. These data provide further evidence for the role of Arg-42 in co-ordinating the substrate.

Kinetic analysis of the UKC510:H20L mutant showed that the replacement of His-20 with Leu caused an 11.5- and 14-fold decrease in catalytic-centre activity for MCA and 2MCPA respectively, with no effect on K_m values. These data suggest that His-20 was not involved in substrate binding, but provides a catalytic advantage. The fact that the UKC510:H20L mutant still demonstrated a significant catalytic activity despite the loss of the His residue implicated as being key in the function of the enzyme indicated that an alternative catalytic site was available in the protein. Apparent kinetic data derived from UKC510:H56L and UKC510:H20L:H56L mutants suggest that, in the absence of His-20, His-56 can act as an alternative catalytic site. The assumption of the important role of His-20 residue as a catalytic site was further supported by the mutation

Scheme 1 Proposed mechanism for halidohydrolase IVa activity, showing proposed role of key amino acid residues

in UKC510:H20L:M24I which did not significantly alter the apparent kinetic parameters of the mutant enzyme when compared with those of UKC510:H20L.

All mutant enzymes demonstrated a high level of immuno-reactivity toward the antibody raised against the wild-type enzyme. This suggests that all mutant enzymes still maintained their main structural domain. However, more critical analyses, such as those measuring c.d., will be required to substantiate this claim.

The results reported here suggest that Arg-42 of *Ps. cepacia* MBA4 Hdl IVa provides the positive charge and forms an electrostatic bond with the acid carboxy group of the substrate and that His-20 provides the nucleophilic attack on the scissile carbon–halogen bond. His-20 and Arg-42 residues are located on the regions of the amino acid sequence 9–20 and 40–50 respectively, which have previously been shown to be highly conserved among the three 2-haloacid halidohydrolase enzymes sequenced to date (Murdiyatmo et al., 1992). The His-20 needs to be in the correct tautomeric form for activity. In order for this to occur it is proposed that the Asp-18, which is also highly conserved in the three halidohydrolases, positions the correct tautomer of His-20 for catalysis in the enzyme–substrate complex and also stabilizes the protonated form of His-20 in the transition-state complex (Scheme 1).

All three halidohydrolases for which sequences have been

published (Schneider et al., 1991; Murdiyatmo et al., 1992) demonstrated chiral specificity, being active only towards the L-isomer of 2MCPA. Amino-acid-modification studies of a wild-type 2-haloacid halidohydrolase (Hdl V) which was active towards both D- and L-2MCPA (results not shown) indicated that cysteine, but not histidine, residues were important for the activity of this enzyme, an observation backed by the inhibitory effect of thiol-group-reactive reagents on the activity of this Hdl V. The N-terminal sequence of this Hdl V over the first 20 amino acids is identical with that predicted for a halidohydrolase from *Ps. putida* PP3 (Thomas, 1990), which is also active towards both D- and L-2MCPA. There was, however, no sequence similarity between these two enzymes and the three L-specific enzymes, and there was no conservation of amino acid sequence in the amino acid 9–20 region. These observations fully support the division of the 2-haloacid halidohydrolases into different groups on the basis of biochemical characteristics (Hardman, 1991), in that the enzymes of *Ps. sp.* CBS3 and *Ps. cepacia* MBA4 have previously been placed in a separate group from those of the *Ps. putida* PP3 enzymes.

U. M. thanks the Agency for Agricultural Research and Development, Indonesia, for a Research Scholarship; W. A. thanks the Government of Indonesia for a Research Fellowship.

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