Catalytic triad of microsomal epoxide hydrolase: replacement of Glu^{404} with Asp leads to a strongly increased turnover rate

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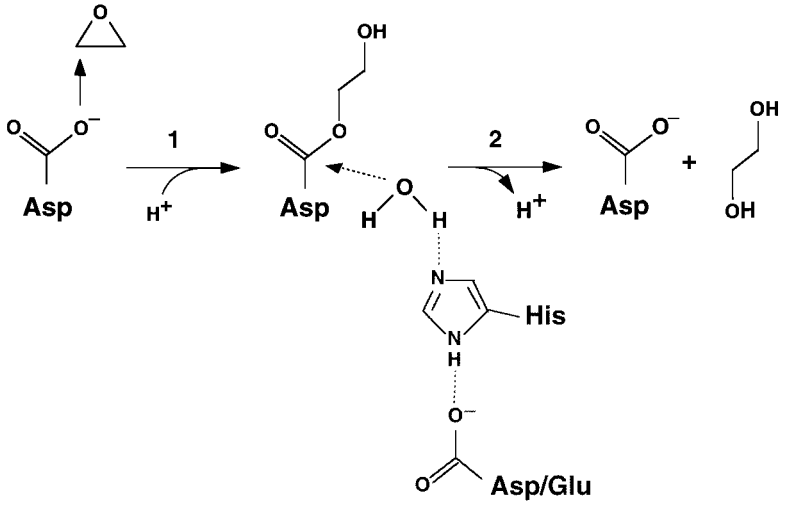
Microsomal epoxide hydrolase (mEH) belongs to the superfamily of α/β-hydrolase fold enzymes. A catalytic triad in the active centre of the enzyme hydrolyses the substrate molecules in a two-step reaction via the intermediate formation of an enzyme-substrate ester. Here we show that the mEH catalytic triad is composed of Asp^{226}, Glu^{404} and His^{431}. Replacing either of these residues with non-functional amino acids results in a complete loss of activity of the enzyme recombinantly expressed in *Saccharomyces cerevisiae*. For Glu^{404} and His^{431} mutants, their structural integrity was demonstrated by their retained ability to form the substrate ester intermediate, indicating that the lack of enzymic activity is due to an indispensable function of either residue in the hydrolytic step of the enzymic reaction. The role of Asp^{226} as the catalytic nucleophile driving the formation of the ester intermediate was substantiated by the isolation of a peptide fraction carrying the ^{14}C-labelled substrate after cleavage of the ester intermediate with cyanogen bromide. Sequence analysis revealed that one of the two peptides within this sample harboured Asp^{226}. Surprisingly, the replacement of Glu^{404} with Asp greatly increased the V_{max} of the enzyme with styrene 7,8-oxide (23-fold) and 9,10-epoxystearic acid (39-fold). The increase in V_{max} was paralleled by an increase in K_m with both substrates, in line with a selective enhancement of the second, rate-limiting step of the enzymic reaction. Owing to its enhanced catalytic properties, the Glu^{404} → Asp mutant might represent a versatile tool for the enantioselective bio-organic synthesis of chiral fine chemicals. The question of why all native mEHs analysed so far have a Glu in place of the acidic charge relay residue is discussed.

INTRODUCTION

Epoxide hydrolases (EC 3.3.2.3) comprise a group of functionally related enzymes that hydrolyse oxirane derivatives to form the corresponding diols [1]. This reaction has important function in the metabolism of foreign compounds: it serves to protect the organism from the potentially genotoxic effect of epoxides [2]. Furthermore the enzymic hydrolysis of endogenous epoxides has implications in several signal transduction cascades [3,4]. During recent years, a growing interest has arisen in the use of epoxide



Scheme 1 Enzymic mechanism of epoxide hydrolysis

In step 1 of the reaction, the catalytic Asp residue opens the epoxide ring by nucleophilic attack at one of the ring carbon atoms. During step 2, a water activated through proton abstraction by a His/acidic residue charge relay system hydrolyses the ester bond between enzyme and product.

Abbreviation used: sEH, soluble epoxide hydrolase; mEH, microsomal epoxide hydrolase.

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hydrolases in bio-organic production processes because these enzymes are versatile catalysts in the stereoselective and enantioselective synthesis of commercially important chemicals [5–7].

The major enzyme involved in the control of xenobiotic metabolites in the mammalian organism is microsomal epoxide hydrolase (mEH), which is expressed in large amounts in the liver and readily hydrolyses a large variety of monosubstituted and *cis*-disubstituted epoxides [2]. Typical examples are many epoxides metabolically formed from polycyclic aromatic hydrocarbons. mEH displays pronounced enantioselectivity and stereoselectivity [8] and thus also qualifies as an interesting candidate for application to bio-organic synthesis. However, its notoriously slow catalytic activity (less than 1 μmol/min per mg of enzyme protein with many of its substrates) severely restricts its value for the latter purpose.

For many years it was believed that direct addition of water to the epoxide, accomplished by base-catalysed activation of a water molecule, was the mechanism by which mEH converts its substrates. This was supported by the initial observation that a single histidine residue was essential for the catalytic process [9]. More recently, His^{431} has been identified as the residue indispensable for the catalytic activity [10,11]. However, a number of observations have meanwhile led to the conclusion that the mEH hydrolyses its substrates by a two-step mechanism via the intermediate formation of a covalent bond between enzyme and substrate [12–16] (Scheme 1). The structural similarity of mEH to the bacterial haloalkane dehalogenase [17] indicates that the enzyme belongs to the superfamily of α/β-hydrolase fold enzymes [18]. These enzymes possess a catalytic triad composed of a catalytic nucleophile and a charge relay system formed by a histidine residue and an acidic residue. In the first step of the enzymic reaction the catalytic nucleophile, usually a serine residue in the esterase/amidase type of enzymes and an aspartic residue in the dehalogenase/epoxide hydrolase type of enzymes, attacks the substrate to form an ester intermediate. Subsequently this is hydrolysed by a water molecule that is activated through proton abstraction by the charge relay system. This mechanism has also just been proved for the soluble epoxide hydrolase (sEH) [19,20], and all components of its catalytic triad have been identified [21].

Here we describe the identification of the members of the mEH catalytic triad by the analysis of recombinant enzymes with mutations of the respective catalytic amino acid residues. Of outstanding interest is the observation that replacing the catalytic Glu^{404} with Asp markedly improves the turnover rate of mEH. The implications of this phenomenon for xenobiotic metabolism and possible biotechnological application of the enzyme are discussed.

EXPERIMENTAL

Isolation of rat mEH cDNA and construction of mutants

The rat mEH cDNA was isolated from a rat liver cDNA library in bacteriophage λgt 11 (RL1023b; Clontech, Palo Alto, CA, U.S.A.) by immunoscreening [22] with a polyclonal rabbit anti-(rat mEH) antiserum [23]. The protein-coding sequence of the obtained fragment was identical with that of the rat mEH cDNA previously isolated by Porter et al. [24]. Site-directed mutagenesis was performed by the method of Tomic et al. [25], essentially as described previously [21]. In brief, mutations were introduced via PCR with a mutation primer carrying a 5′-terminal *Bbs*I site (Table 1) that was used together with a suitable counter primer to amplify a fragment harbouring the desired mutation. A second amplification reaction was performed in a similar manner to create an adapter fragment by using an adapter primer also carrying a 5′-terminal *Bbs*I site. The *Bbs*I sites in the two different primers were designed so as to allow the reconstitution of the original open reading frame of the mEH on ligation of the two corresponding *Bbs*I-restricted PCR fragments. Each fragment was digested with an additional appropriate restriction enzyme to allow the integration of the construct into a cloning vector [pBluescript II SK (+); Stratagene, La Jolla, CA, U.S.A.]. The incorporation of the desired mutation and the absence of unwanted additional sequence alterations were confirmed by dideoxy sequencing [26]. The respective mutations were transferred into the complete mEH open reading frame by exchange of either a *Msc*I–*Stu*I fragment for Asp^{226} mutants (note that a second *Stu*I site in the rat mEH cDNA is blocked due to methylation by DNA cytosine methylase and was not used by *Stu*I under our experimental conditions) or a *Hpa*I–*Hin*dIII fragment in all other mutants. The resulting constructs were transferred as *Bgl*II/*Eco*RI fragments into the *Bam*HI/*Eco*RI site of the galactose-inducible expression vector pYeDP60 [27] (pYeDP60-mEH is shown in Figure 1 as a prototype of the resulting constructs).

Expression of wild-type and mutant mEH in yeast

For production of the recombinant proteins, competent cells of the *Saccharomyces cerevisiae* strain W303-1B were transformed with different expression constructs by the method of Gietz et al. [28]; expression of the recombinant proteins was accomplished by induction with galactose [27]. At a final D_{600} of approx. 20, cells were pelleted by centrifugation and resuspended in 1/25 volume of TKE buffer [50 mM Tris/HCl/100 mM KCl/5 mM EDTA (pH 7.4)], then microsomal membranes were prepared from the cells as described [27].

Assay procedures

Epoxide hydrolase activity towards styrene 7,8-oxide and *trans*-stilbene oxide were assayed as described by Oesch [29] and Schladt et al. [30] respectively. Epoxide hydrolase activity towards 9,10-epoxystearic acid and cholesterol 5,6-epoxide were determined as described by Müller et al. [16]. Protein content was quantified by the method of Bradford [31]. Immunoblot analysis was performed after SDS/PAGE [32] and subsequent electrotransfer of the samples to nitrocellulose sheets [33], with the use of the above polyclonal rabbit anti-(rat mEH) antiserum as the specific probe. Detection of the immunocomplex was accomplished with an alkaline phosphatase-coupled anti-(rabbit IgG) antibody (Sigma, St. Louis, MO, U.S.A.), with Nitro Blue Tetrazolium and 4-bromo-5-chloro-3-indolyl phosphate as the chromogenic substrates. Detection of the covalent intermediate formed between 9,10-epoxy[^{14}C]stearic acid and mEH was performed essentially as described previously [16]. Quantification of immunosignals and autoradiographic signals was accomplished by digitization of the membranes or autoradiographs with an EagleEye II still video system (Stratagene, La Jolla, CA, U.S.A.) and subsequent processing with the Gel Plotting Macro of the NIH Image analysis software package (version 1.52) written by Wayne Rasband.

Peptide analysis

mEH was isolated from the livers of *trans*-stilbene oxide-treated rats as described earlier [34]. Pure enzyme (1 mg) was covalently labelled with 9,10-epoxy[^{14}C]stearic acid as described [16] and then subjected to cleavage with CnBr in 70 % (v/v) formic acid in a final volume of 22 μl under an argon atmosphere for 24 h. After the addition of 180 μl of water, the sample was evaporated

Table 1 Oligonucleotides used for the introduction of specific mutations

The nucleotide sequences of the mutation primers and the matching adapter primers are given. The *Bbs*I recognition sites are shown in bold letters, and mutated residues are shown in bold italic letters. The part of the sequence that is removed by the digestion with *Bbs*I is presented in lower-case letters. The resulting 5′-cohesive ends are underlined.

	Mutation primer	Adapter primer
$Asp^{226} \rightarrow Asn$	atg**gaagac**cc<u>CCAGT***T***</u>CCCGCCT	caa**gaagac**ga<u>CTGG</u>GGGTCCCTCAT
$Asp^{226} \rightarrow Gly$	atg**gaagac**cc<u>CCAG***C***</u>CCCCGCCT	caa**gaagac**ga<u>CTGG</u>GGGTCCCTCAT
$Asp^{226} \rightarrow Ser$	atg**gaagac**cc<u>CCAG***CT***</u>CCCGCCT	caa**gaagac**ga<u>CTGG</u>GGGTCCCTCAT
$Glu^{388} \rightarrow Ala$	tgg**gaagac**aa<u>ACATG***C***</u>GGGGATGAAGG	ttc**gaagac**tc<u>ATGT</u>TTATGGACCATGAT
$Glu^{404} \rightarrow Ala$	tag**gaagac**ct<u>TCCG***C***</u>GCTACTGCAT	ttg**gaagac**ct<u>CGGA</u>AGGGAAGGCTGA
$Glu^{404} \rightarrow Asp$	tag**gaagac**ct<u>TCCGA***T***</u>CTACTGCAT	ttg**gaagac**ct<u>CGGA</u>AGGGAAGGCTGA
$Glu^{410} \rightarrow Ala$	tag**gaagac**cc<u>CCAG***C***</u>AAAGTGGGTGA	tac**gaagac**tt<u>CTGG</u>GGCATGCAGTAG
$His^{431} \rightarrow Gln$	tag**gaagac**gc<u>AGCAAA***T***</u>TGGCCCC	tag**gaagac**tt<u>TGCT</u>GCCTTTGAA

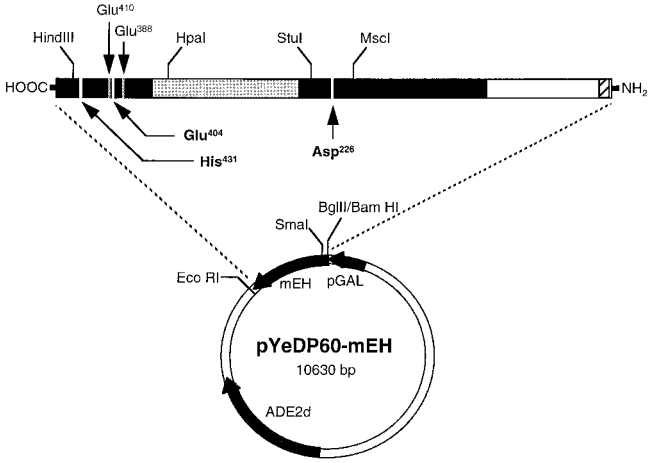

Figure 1 Expression construct for the recombinant production of mEH mutants in *S. cerevisiae*

The construction of the vector is described in the Experimental section. Restriction sites used for recombination *in vitro* as well as the positions that were engineered by site-directed mutagenesis are indicated. The dark boxes in the mEH gene identify the regions that code for the potential α/β-hydrolase fold domain that is predicted to be interrupted by a cap domain (grey box). The hatched box close to the N-terminus/5′ end of the gene indicates the single membrane anchor of the enzyme. The gene is under the control of the galactose-inducible *pGAL* promoter. The *Ade2d* gene in the plasmid backbone is required for the selection of recombinant clones of the Ade-deficient yeast strain W303.

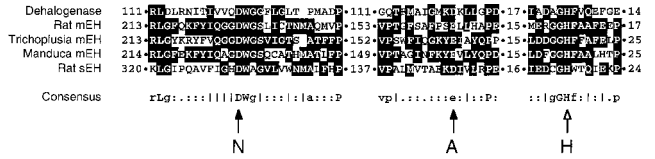

Figure 2 Sequence comparison between mEHs from rat, *Manduca sexta* and *Trichoplusia ni*, soluble epoxide hydrolase and haloalkane dehalogenase over the sequence context of the (potential) catalytic triads

Symbols in the consensus indicate the following: capital letters, identical residue in all sequences; small letters, identical residue in four of the five sequences; vertical bars, identical residue in three of the five sequences; colon, identical residue in two of the five sequences; dot, different residues throughout the five sequences. The arrows indicate the positions of the identified or proposed catalytic triad residues. Sequences correspond to the SwissProt data files P22643 (dehalogenase from *Xanthobacter autotrophicus*), P07687 (rat mEH) and P80299 (rat soluble EH), and the EMBL data files U46682 (*Manduca sexta* mEH) and U73680 (*Trichoplusia ni* mEH).

to dryness in a Speed-Vac device and dissolved in acetonitrile/water (1:10, v/v). Peptides, in four portions of equal size were separated on a Vydac C_{18} column (4.6 mm × 250 mm) with a water/acetonitrile gradient (10–75%, v/v) containing 0.1% (v/v) trifluoroacetic acid. Radioactivity in the fractions eluted was monitored by liquid-scintillation counting. The fractions containing detectable amounts of ^{14}C were subjected to N-terminal sequencing via Edman degradation.

RESULTS

Identification of the catalytic triad components

On the basis of sequence alignments of mEH with other haloalkane dehalogenase-related enzymes (Figure 2) [13,35,36], we selected candidate amino acid residues for the members of the catalytic triad of the enzyme. We then constructed yeast clones that, under permissive conditions, expressed mutant mEH proteins with single amino acid residue substitutions for the putative members of the catalytic triad. Immunoblot analysis performed on microsomal preparations from these clones

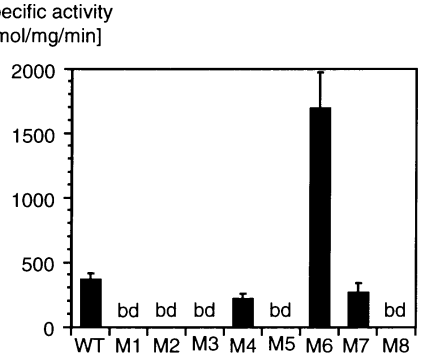

Figure 3 Catalytic activity of rat mEH mutants after recombinant expression in *S. cerevisiae*

Mutant mEH proteins were expressed and analysed as described in the Experimental section by using styrene 7,8-oxide as substrate. Enzymic activity was calculated for each mEH mutant on the basis of immunoquantification of the expressed recombinant protein. Error bars represent the S.D. for at least four independent determinations. Abbreviations: WT, wild-type enzyme; M1, $Asp^{226} \rightarrow Asn$; M2, $Asp^{226} \rightarrow Gly$; M3, $Asp^{226} \rightarrow Ser$; M4, $Glu^{388} \rightarrow Ala$; M5, $Glu^{404} \rightarrow Ala$; M6, $Glu^{404} \rightarrow Asp$; M7, $Glu^{410} \rightarrow Ala$; M8, $His^{431} \rightarrow Gln$; bd, below detection.

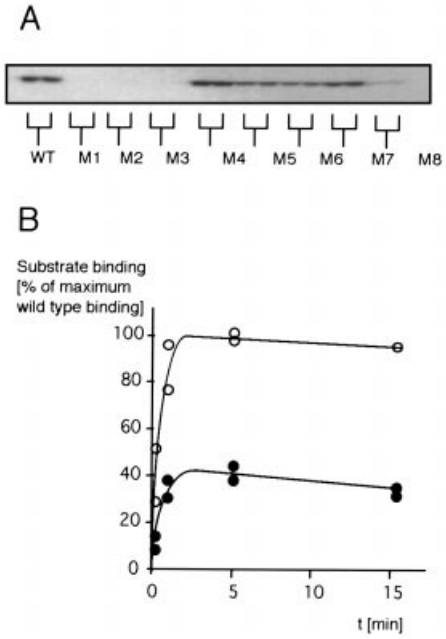

Figure 4 Proof of correct folding of the mutants by trapping the ester intermediate

(**A**) Autoradiograph of the ester intermediate formed between the different recombinant mEH mutants and 9,10-epoxy[^{14}C]stearic acid. The mutants M1–3, which lack the catalytic nucleophile, do not show binding of radioactive substrate. All other mutants display epoxystearic acid binding to a varying degree. Note the compromised binding capacity of the His^{431} → Gln mutant (M8). Abbreviations: WT, wild-type enzyme; M1, Asp^{226} → Asn; M2, Asp^{226} → Gly; M3, Asp^{226} → Ser; M4, Glu^{388} → Ala; M5, Glu^{404} → Ala; M6, Glu^{404} → Asp; M7, Glu^{410} → Ala; M8, His^{431} → Gln. (**B**) Time course of the substrate binding of wild-type enzyme and the His^{431} → Gln mutant (M8). The amount of bound radioactivity was determined by densitometric analysis of the respective autoradiograms and subsequent normalization to the amount of immunoreactive protein in the respective sample. Although no diffference is observed between the two proteins in the velocity of covalent substrate binding, it becomes evident that the His^{431} → Gln mutant preparation binds only 40% of the amount of substrate bound to the wild type, indicative of a substantial amount of the mutant protein's being improperly folded.

revealed an mEH protein expression level of approx. 0.2 % of the microsomal protein. Duplicate expression from the same clone yielded remarkably similar results in terms of recombinant protein content, the variation usually not exceeding 20 %. Some difference in the mEH expression level was observed between the different clones, but rarely exceeded a factor of 2. Replacement of Asp^{226}, the predicted catalytic nucleophile of rat mEH, with Asn, Gly or Ser led to an enzyme completely unable to hydrolyse the model substrate styrene oxide (Figure 3). Similarly, replacement of His^{431} with Gln inactivated the enzyme, as observed already by Bell and Kasper [11]. Glu^{404} evolved as the third member of the catalytic triad because its replacement with Ala also abolished enzymic activity. In contrast, the replacement of either Glu^{388} or Glu^{410} did not significantly influence the protein's catalytic properties.

To assess the structural integrity of the mutant proteins we trapped the covalent intermediate formed between enzyme and the radiolabelled substrate and revealed it by autoradiography (Figure 4A). Incubation of the enzyme with 9,10-epoxy[^{14}C]stearic acid and subsequent analysis of the denatured protein revealed covalent substrate binding of the wild-type mEH and all mutants under investigation, with the exception of those that lacked Asp^{226}, i.e. the residue that takes part in the formation of the ester intermediate. However, quantitative differences were observed. In particular, the His^{431} → Gln mutant displayed strongly impaired substrate binding compared with the wild type. To determine whether this was due to altered reaction kinetics, we recorded the time course of the formation of the covalent intermediate for the wild-type enzyme and the His^{431} → Gln mutant (Figure 4B). In this analysis, the curve recorded for the His mutant paralleled that for the wild-type enzyme, both reaching maximum substrate binding after approx. 1 min. The His mutant, however, bound only approx. 40 % of the amount of substrate bound by the wild-type enzyme, as calculated after correction for the slightly lower amount of mEH protein expressed from the His mutant (79 % of the wild-type protein expression level).

To confirm or disprove the role of Asp^{226} as the catalytic nucleophile we subjected the trapped ester intermediate to cyanogen bromide cleavage and subsequently isolated the peptide fraction carrying the radioactive label. A single peak was obtained that was eluted from the column at 60 % (v/v) acetonitrile. Edman degradation of the respective pooled fractions revealed the presence of two free N-termini of approximately equal abundances in the sample. The sequence obtained over ten cycles was [T,V][H,R][G,L][G,W][F,P][G,Q][K,S][F][Y][E,I]. On comparison with the known primary sequence of rat mEH, this turned out to be a composite of the sequences VHGWPGSFYE (residues 147–156) and TRLGFQKFYI (residues 213–222). The latter directly precedes the predicted catalytic nucleophile Asp^{226}.

Analysis of potentially functional mutants

Because most other α/β-hydrolase fold enzymes have an Asp residue in place of the acidic residue of the charge relay system, we replaced the corresponding Glu^{404} of mEH with Asp. In a first comparative analysis, the resulting mEH mutant displayed a significantly enhanced enzymic activity towards styrene oxide in comparison with the wild-type enzyme (Figure 3). The rate of substrate conversion followed Michaelis–Menten kinetics up to approx. 500 μM. Extrapolation revealed a 23-fold higher V_{max} for the mutant enzyme (Figure 5A) (8.5 μmol/min per mg of mEH, compared with 365 nmol/min per mg of mEH for the wild-type enzyme). The apparent K_m of the enzyme increased by 32-fold (to 2.8 mM, compared with 88 μM for the wild-type enzyme). At low substrate concentrations (less than 30 μM), this resulted in a slightly higher turnover number for the wild-type enzyme, reaching 1.4-fold that of the mutant enzyme at an infinitesimal substrate concentration.

To investigate whether the catalytic activity of mEH was elevated in general by the Glu^{404} → Asp change, *cis*-9,10-epoxystearic acid was chosen as a second substrate with low structural similarity to styrene oxide. As with styrene oxide, a marked increase in enzymic activity was observed towards this compound (Figure 5B). The relation between substrate concentration and turnover again followed Michaelis–Menten kinetics up to at least 30 μM, the highest concentration applied in the analysis for practical reasons. A calculated 39-fold increase in V_{max} from 10 nmol/min per mg of mEH to 390 nmol/min per mg was accompanied by a 31-fold increase in the apparent K_m, from 7 to 215 μM.

To establish whether the Glu^{404} → Asp change influenced the strong preference of mEH for exclusively *cis*-substituted oxirane derivatives, two *trans*-substituted epoxides, namely *trans*-stilbene oxide and cholesterol 5α,6α-epoxide, were tested as substrates for the mutant. However, no detectable turnover of either compound was obtained on extended incubation with the mutant enzyme.

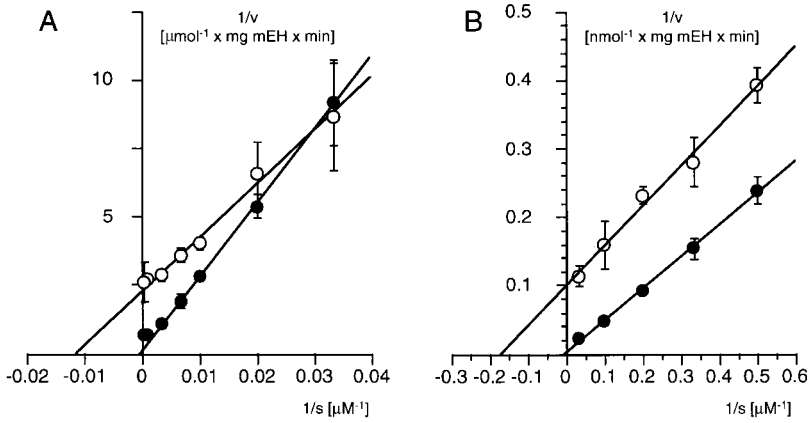

Figure 5 Kinetic analysis of the hyperactive mutant Glu^{404} → Asp

Lineweaver–Burk plots of the substrate dependence of the catalytic activity of the wild-type enzyme (○) and the Glu^{404} → Asp mutant (M6; ●) towards styrene 7,8-oxide (**A**) and 9,10-epoxystearic acid (**B**). V_{max} was 365 nmol/min per mg for the wild type and 8420 nmol/min per mg for M6 (**A**), and 10 nmol/min per mg for the wild type and 388 nmol/min per mg for M6 (**B**). K_m was 87.5 μM for the wild type and 2770 μM for M6 (**A**), and 6.8 μM for the wild type and 213 μM for M6 (**B**). The r^2 value for the slope was 0.991 for the wild type and 0.999 for M6 (**A**), and 0.990 for the wild type and 0.998 for M6 (**B**).

Serine is the catalytic nucleophile of many esterase/amidase types of enzymes. The Asp^{226} → Ser mutant was constructed to determine whether a simple one-residue exchange could turn an epoxide hydrolase into an esterase. We used two chromogenic substrates to monitor the potential esterase activity. These were chosen on the basis of their structural similarity to mEH substrates. Nitrophenyl acetate is sterically similar to nitrostyrene oxide, whereas the nitrophenyl stearate was used because of its potential for increased hydrophobic interaction with the mutant enzyme. However, both substrates were not detectably hydrolysed by the mutant enzyme.

On the basis of the reports for soluble EH and haloalkane dehalogenase that the replacement of the catalytic nucleophile Asp residue with Asn leads to an initially inactive enzyme that self-regenerates after a period of several days to a completely active enzyme, we constructed the respective Asp^{226} → Asn mutant. Incubation of a microsomal preparation from the respective recombinant yeast at 37 °C in TKE buffer for up to 4 weeks did not result in the expected regeneration, whereas a similar preparation of the wild-type enzyme treated in parallel retained its full activity over this period of time. Immunoblotting of the two different preparations at the end of the experimental period did not show any sign of mEH protein degradation, indicating that the mutant enzyme had remained intact yet inactive. Similar experiments were performed with a double mutant Asp^{226} → Asn/Glu^{404} → Asp because the Glu^{404} → Asp change had obviously increased the velocity of the enzymic hydrolysis by mEH (see the Discussion section). However, with this protein, too, no self-activation could be detected.

DISCUSSION

Catalytic triad of mEH

A sequence comparison between mEH, soluble EH and haloalkane dehalogenase revealed candidate residues for the catalytic triad of mEH (Figure 2). Although the catalytic nucleophile could be predicted with high accuracy to be Asp^{226}, and the catalytic histidine His^{431} has already been identified [11], the acidic amino acid residue of the charge relay system could not be predicted with sufficient accuracy. Lacourciere and Armstrong [14] suggested Asp^{352} as the most likely candidate, whereas Beetham et al. [35] favoured Glu^{404}. A compelling argument that Glu^{404} is the missing component of the charge relay system was given by a recent sequence alignment of 21 enzymes structurally related to haloalkane dehalogenase, in which the distance between the catalytic histidine residue and the acidic component of the charge relay system was invariably between 25 and 35 residues [36]. However, it should be noted that the vast majority of epoxide hydrolase/haloalkane dehalogenase-related α/β-hydrolase fold enzymes has an aspartic residue in place of the acidic amino acid of the charge relay system. Our results with the inactive mutant enzymes obtained in the present study show clearly that His^{431} and Glu^{404} form the charge relay system of the mEH catalytic triad, because a separate replacement of these amino acid residues with non-functional substitutes leads to enzymes that can still perform step 1 of the enzymic reaction as demonstrated by covalent binding of the substrate, but not step 2 of the reaction as evidenced by the lack of product formation. The importance of monitoring the structural integrity of mutant proteins in this type of experiment is underlined by the observation that a significant proportion of the His^{431} → Gln mutant is unable to bind the substrate covalently and is therefore obviously incorrectly folded. A similar result has recently been obtained by Rink et al. [37] by replacing the catalytic His in the triad of a bacterial epoxide hydrolase. In their experiments, approx. 70 % of their His^{275} → Arg mutants were structurally compromised. However, in both cases the amount of correctly folded mutant protein is still sufficient to draw valid conclusions from the lack of activity of the respective enzyme preparation.

Our results with the Asp^{226} mutants are in line with this residue's being the catalytic nucleophile of the enzyme, although no unequivocal proof for the correct folding of the mutant proteins could be provided, because the covalent binding of the substrate to the enzyme is dependent on a functional catalytic nucleophile. We tried to support the role of Asp^{226} further by analysing the self-activation potency of an Asp^{226} → Asn mutant. Despite the surprising stability of the enzyme in yeast microsomal membranes (no detectable change in activity of the wild-type enzyme at 37 °C over 1 month) we did not find any detectable enzymic activity with the mutant protein that would have been indicative of autocatalytic deamidation. This is in apparent contradiction of results obtained recently by Armstrong and

colleagues, who found self-activation of an Asp^{226} → Asn mutant with a reactivation half-life of 9.3 days [38]. This discrepancy might be because the latter work was performed with purified enzyme in solution at slightly higher pH (pH 8), whereas we assessed self-activation in yeast microsomes, i.e. in a lipid environment, at pH 7.4.

We could provide additional evidence that Asp^{226} is the catalytic nucleophile by sequence analysis of the peptide that carried the radioactive substrate, after degradation of the labelled protein with cyanogen bromide. One of the two sequences obtained from the positive fraction was the N-terminus of that one of the expected 13 peptides that harboured the predicted catalytic nucleophile, i.e. Asp^{226}.

While the present paper was in preparation, Armstrong and colleagues [38,39] reported on the analysis of the mEH catalytic triad with a similar approach to that described here. Although their results are in agreement with our observations with respect to the catalytic nucleophile and the catalytic histidine residue, they propose a somewhat different setting for the acidic residue of the charge relay system. On the basis of their experimental data, and by analogy with a recent proposal by Janssen and co-workers [37] for the active site of a bacterial epoxide hydrolase, they conclude that Glu^{404} and Glu^{376} in concert support the histidine residue in proton abstraction. On replacing either of these residues with glutamine, they find a substantial but not a complete loss of enzymic activity with their mutant enzyme. With the Glu^{404} → Gln mutant, they can still detect up to 20 % of the wild-type activity, depending on the substrate. In contrast, our replacement of Glu^{404} with Ala leads to a completely inactive enzyme. We disagree with the conclusions of Armstrong and co-workers on this specific point for the following reasons: (1) the retention of some catalytic activity with the Glu^{404} → Gln mutant can be explained by the ability of Gln to substitute for Glu in hydrogen-bonding with His^{431}; (2) the loss of activity with the Glu^{376} → Gln mutant, in contrast, might be due to a high proportion of misfolded mutant protein. The latter explanation is in line with the observations by Tzeng et al. [39] that the mutant protein is highly unstable under their standard purification conditions and does not show appreciable fluorescence quenching on interaction with the substrate, usually a measure of the formation of the covalent intermediate during step 1 of the enzymic hydrolysis of epoxide. This step should remain largely unaffected by modifying the charge relay system. Finally, the fact that the K_m for the Glu^{376} → Gln mutant is not decreased proportionally to V_{max} indicates, for reasons detailed below, that the hydrolytic step of the enzymic reaction does not depend significantly on Glu^{376}. Therefore we conclude that the catalytic triad of mEH consists of Asp^{226}, Glu^{404} and His^{431} only.

Mutant Glu^{404} → Asp, a better mEH?

The striking observation that the replacement of Glu^{404} with Asp increases the enzymic activity by approx. 20–40-fold with two structurally very different substrates is particularly interesting in view of the fact that all mEHs analysed so far, i.e. those from mammals and insects, have a glutamic residue at the equivalent position. Why is the apparently more efficient variant obviously avoided in Nature? A simple answer might be that it is in fact not more efficient. The concomitant change in K_m provides a clue to this: in enzymic reactions involving a covalent intermediate, K_m is defined by $K_m = K_d k_2/(k_1+k_2)$ [40], where K_d is the dissociation constant for the Michaelis–Menten complex, k_1 is the rate constant for the formation of the covalent intermediate and k_2 is the rate constant for the formation of the final product. Thus if $k_1 \gg k_2$, K_m is directly proportional to k_2. The fact that K_m and V_{max} are increased in parallel by the replacement of Glu^{404} with Asp suggests that, at least with the substrates under investigation, k_2 is indeed much smaller than k_1 and is selectively enhanced by the modification, being in line with the observation by Tzeng et al. [15] that k_1 is about three orders of magnitudes higher than k_2 for the turnover of glycidyl 4-nitrobenzoate enantiomers. However, because the formation of the enzyme-substrate ester represents the actual detoxification step in the reaction, the enhanced hydrolysis of the intermediate does not necessarily present an advantage, especially under conditions in which the enzyme is in sufficient abundance over its substrates, as is often the case with mEH, whose concentration in human liver is between 20 and 50 μM.

Another possible explanation for the strict requirement of a Glu residue as the acidic charge relay residue might be that a gain in function by a Glu → Asp change might have adverse effects for the organism. The strongly enhanced activity of the Glu^{404} → Asp mutant with fatty acid epoxides, for instance, might lead to an enhanced formation of the respective diols, some of which are under consideration as important signal molecules in pathophysiological processes [4].

In contrast with the apparent disfavour of Nature towards the 'rapid' mutant, biotechnologists might consider taking full advantage of the enhanced turnover rate of the recombinant enzyme. In bio-organic processes, the proportional increases in K_m and V_{max} do not pose a problem because high substrate concentrations are desirable. The fact that both styrene 7,8-oxide and 9,10-epoxystearic acid are hydrolysed much faster by the mutant than by the wild-type enzyme suggests that turnover rates of a broad range of substrates for the enzyme might benefit from the mutation.

In conclusion, our results prove that Asp^{226}, Glu^{404} and His^{431} constitute the catalytic triad in mEH. The replacement of Glu^{404} with Asp leads to an enzyme with a strongly enhanced catalytic activity that has, however, not yet been found in Nature. Despite the apparent disadvantage that this mutant might therefore represent for living organisms, it could become a valuable tool for bio-organic chemists.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 519) and the DG XII of the European Community under the fourth framework programme (PL950005, Biotechnology).

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Received 23 July 1998/23 September 1998; accepted 14 October 1998