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ORIGINAL PAPER

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Modification of the enantioselectivity of two homologous thermophilic carboxylesterases from *Alicyclobacillus acidocaldarius* and *Archaeoglobus fulgidus* by random mutagenesis and screening

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Abstract The esterase genes *est2* from *Alicyclobacillus acidocaldarius* and *AF1716* from *Archaeoglobus fulgidus* were subjected to error-prone PCR in an effort to increase the low enantioselectivity of the corresponding enzymes EST2 and AFEST, respectively. The model substrate (*RS*)-*p*-nitrophenyl-2-chloropropionate was chosen to produce (*S*)-2-chloropropionic acid, an important intermediate in the synthesis of some optically pure compounds, such as the herbicide mecoprop. In the case of EST2, a single mutant, Leu212Pro, was obtained showing a slightly enhanced preference toward the (*S*) substrate; in the case of AFEST, a double mutant, Leu101Ile/Asp117Gly, was obtained showing an increased preference in the opposite direction. The 3-D structures of the EST2 and AFEST enzymes were analyzed by molecular modeling to determine the effects of the mutations. Mutations were positioned differently in the structures, but in both cases caused small modifications around the active site and in the oxyanion loop.

Key words Carboxylesterase · Enantioselectivity · *Archaeoglobus fulgidus* · *Alicyclobacillus acidocaldarius* · Modeling

Introduction

Among the properties of enzymes that are amenable to manipulation by organic chemists is the capacity to discriminate between the two enantiomers of a substrate. In the past, the reaction of one enantiomer over another could be favored by the modification of reaction conditions (medium engineering; Carrea and Riva 2000) or by the modification of the substrate by the addition of protective groups of different size and/or polarity (substrate modification; Faber et al. 1993). More recently, it has become feasible to choose a different catalyst with enhanced specificity through "environmental cloning," especially from extreme habitats (Frederickson 1999).

In the last few years, directed evolution has surged as a tool to create new biodiversity, and it has been demonstrated that it is possible to modify the activity, stability, pH optimum, and specificity of different enzymes (Petrounia and Arnold 2000). However, very few researchers have reported enantioselectivity modifications. Henke and Bornscheuer (1999) were able with a single round of mutation and screening to increase the enantioselectivity of a *Pseudomonas fluorescens* esterase from E = 3.5 to E = 5.2 – 6.6. Jaeger and coworkers (Liebeton et al. 2000) reported a 25-fold increase of enantioselectivity for a mutated lipase from *Pseudomonas aeruginosa* PA01 toward the (*S*) enantiomer of *p*-nitrophenyl-2-methyldecanoate with respect to the wild type. Five mutations were introduced into the sequence and were found to be located mostly in loops. Arnold and coworkers (May et al. 2000) reported the use of directed evolution to improve the production of L-methionine from D,L-5-(2-methylthioethyl)hydantoin in a whole-cell system of recombinant *Escherichia coli*. This was accomplished by inverting the enantioselectivity of a hydantoinase from *Arthrobacter* sp. DSM 9771 from a D-selectivity (40% ee) to a moderate L-preference (20% ee at 30% conversion). Only one amino acid substitution was sufficient to invert the enantioselectivity and to improve five-fold the specific activity. These are, to our knowledge, the only studies reported so far.

We selected as model systems for our studies of directed evolution an esterase from the thermophilic eubacterium *A. acidocaldarius* (EST2) and one homologous from the hyperthermophilic archaeon *A. fulgidus* (AFEST). (Hyper)thermophilic esterases are generally considered an important addition to the catalogue of enzymes for

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biotransformations because of their intrinsic stability under the harsh conditions of industrial plants compared with their mesophilic counterparts. These two enzymes have previously been overexpressed, purified, and extensively characterized by our group (Manco et al. 1998, 2000a, b, 2001). The enzymes have similar residue numbers (311 and 310 for AFEST and EST2, respectively), and both are monomeric (Manco et al. 1998, 2000b). The 3-D structures of EST2 and AFEST, complexed with an inhibitor, have been recently obtained (De Simone et al. 2000, 2001). Thus, the system was interesting because there was the opportunity to map the obtained mutations on the 3-D structures and to analyze the effects of mutations.

Materials and methods

Substrate synthesis

(*R*)-2-Chloropropionic acid (99%, Aldrich) or (*S*)-2-chloropropionic acid (99%, Aldrich) (13.80 mmol) and *p*-nitrophenol (27.60 mmol) were reacted in 18 ml of chloroform in the presence of 1,3-dicyclohexylcarbodiimide (15.20 mmol) and 4-(dimethylamino)pyridine (0.14 mmol) for 5 h at 0°C and for 12 h at room temperature. The reaction mixture was purified by flash chromatography on a silica gel Merck 60 (230–400 mesh) column using chloroform–petroleum ether (1 : 1) as the eluent. Purification gave 7.61 mmol (55% yield) of (*R*)- and 8.03 mmol (58% yield) of (*S*)-*p*-nitrophenyl-2-chloropropionate. Analysis calculated for $C_9H_8ClNO_4$: C, 47.08; H, 3.51; Cl, 15.44; N, 6.10; O, 27.87. Found for the (*R*)-enantiomer: C, 47.17; H, 3.50. Found for the (*S*)-enantiomer: C, 47.21; H, 3.48. $[\alpha]_D^{25}$ for the (*R*)-enantiomer, +33.5 (c 2, isopropanol); $[\alpha]_D^{25}$ for the (*S*)-enantiomer, –33.3 (c 2, isopropanol).

Strains and plasmids

The expression vectors utilized were pT7-SCII-AG (Manco et al. 2001), which was prepared starting from vector pT7-SCII-GM1 (Manco et al. 1998) and harbored the *est2* gene, and pT7-SCII-GM2 (Manco et al. 2000b) harboring the *AF1716* gene. *E. coli* Top 10 (Invitrogen) was used as the host for plasmid preparation and for gene expression. In fact, to obtain enough recombinant clones, we took advantage of the higher transformation efficiency of this strain coupled with the basal expression of the enzymes, which in the absence of isopropyl thiogalactoside (IPTG) induction was satisfactory for our screening purposes.

Random mutagenesis by error-prone PCR

Mutations were introduced in the *AF1716* and *est2* genes by using the Diversify kit (Clontech). This method of mutagenesis provides control over the level of random mutation by independently varying the amounts of manganese and dGTP in the PCR reaction using *Taq* polymerase. The mutation rate can be set to introduce from two to eight mutations per 1,000 bp. Two pairs of oligonucleotides were used in the PCR reactions to amplify the two genes under conditions that were chosen to introduce two mutations per 1,000 bp.

For *est2*, the oligonucleotides were Nter(+) 5′-GGCG ACCCATATGCCGCTCGATCCC and Cter(–), 5′-TTGG ATCCGCCTTTTGGTCAGG-3′. The Nter(+) oligonucleotide contains an *Nde*I cutting site (underlined), which, together with a *Bgl*II unique site at the C-terminus of the gene, allowed the recloning of the mutated *est2* gene (digested with *Nde*I and *Bgl*II) in the plasmid pT7-SCII-MG1, which was digested with the same restriction enzymes and separated from the wild-type gene on an agarose gel. For the saturation mutagenesis, the oligonucleotides used were Leu212(+), 5′-CCGGCGGCATGATGNNNTGGTT CCGGGATC-3′; and Leu212(–), 5′-GATCCCGGAACC ANNNCATCATATGCCGCCGG-3′. The two external oligonucleotides were Ser(+), 5′-TCGGCGGAGACGGCG CCGGAGGGAA-3′; and Cter(–), 5′-TTGGATCCGCC TTTTGGTCAGG-3′. For *AF1716*, the oligonucleotides were AFnternew(+), 5′-GGAGATATACATATGCTTGAT ATGCCAATCGACCCTG; and AFcternew(–), 5′-GATA AGCTTGGGCTGCAGAAGGCCATCGGG. The two oligonucleotides introduced *Nde*I and *Pst*I restriction sites at the N- and C-terminus, respectively. The PCR product and the plasmid pT7-SCII-GM2 were digested with *Nde*I and *Pst*I, and the mutated *AF1716* gene was recloned in the plasmid made free of the wild-type gene. The PCR conditions were as follows: 4 min at 94°C and 30 cycles of 1 min at 92°C, 1 min at 50°C, and 1 min at 72°C.

Screening

Mutated genes were transformed in Top10 *E. coli* cells and the obtained colonies were transferred by sterile toothpicks to 96-well microtiter plates. The colonies were dissolved in 200 µl of Luria-Bertani (LB) medium containing ampicillin (0.1 mg/ml), and the plates were incubated overnight at 37°C. The following day, the colonies were diluted (usually 30-fold in 25 mM Tris/HCl, pH 7.5) in a new microtiter plate and, after transfer to a nitrocellulose filter, assayed with β-naphthyl acetate, as previously described (Manco et al. 2001). Only colonies active toward β-naphthyl acetate were retained for further analyses. From the dilution plate, 10- to 20-µl amounts of cultures were transferred to new plates for the enzymatic assays for (*R*)- or (*S*)-*p*-nitrophenyl-2-chloropropionate. The assays were performed as follows: 10–20 µl of cells were added to 200 µl of 0.4% gum arabic, 0.1% Triton X-100, 30 µl of 0.4 M citrate buffer at pH 5.0. Plates were incubated for 5 min at 70°C to allow for cellular lysis; then, after cooling, the reaction was started at room temperature by the addition of 25 µl propan-2-ol containing 20 mM (*R*) or (*S*)-*p*-nitrophenyl-2-chloropropionate substrate. The use of *p*-nitrophenol as the alcohol moiety was required to facilitate the screening by allowing a spectrophotometric reading at 405 nm of the *p*-nitrophenolate anion. Preliminary analyses indicated that the substrate was particularly unstable under the optimal conditions for

the esterase assay, namely pH 7.0–7.5 and 70°C, probably because of the electron withdrawing action exerted by the halogen atom linked to the carbon in the α-position of the ester bond, which facilitates hydrolysis. To overcome the problem of spontaneous hydrolysis, the clones were assayed at room temperature with a citrate buffer at pH 5.0. Although these were suboptimal conditions for the enzymes, it was still necessary to dilute cell cultures 30-fold before the assay, due to the high catalytic rate of enzymes even at room temperature (Manco et al. 1998, 2000a) and the indicated pH. Actually, the pH optimum of these enzymes is quite broad and does not pose special problems. Obviously, enantioselectivity might depend on pH, and differences between the wild-type and mutants could be amplified at different pHs, but this is a secondary problem. Our goal here was to find mutants with altered enantioselectivity at pH 5 and room temperature with a simple assay, and to retest the enantioselectivity at different pHs in a future study.

At intervals, absorbance at 405 nm was automatically read in a Microplates Reader Apparatus (Bio-Rad) and initial velocities calculated (V_R or V_S). Only clones that deviated more than 2σ from the mean V_S/V_R ratio in a given direction or in the opposite one with respect to the values of the wild types were reassayed to confirm the results. Only clones found to still deviate from the wild-type values in this second assay were cultured, the enzymes partially purified by incubating crude extracts for 20 min at 65°C, and precipitated proteins removed by 10 min of centrifugation at 8,000 *g*. Enzymes were assayed with a DU600 spectrophotometer (Beckman, Fullerton, CA, USA) by using the same conditions outlined above, except that the assay volume was 1 ml, Triton X-100 and gum arabic were omitted, and cuvettes were thermostated at 37° or 50°C, as indicated.

The theory of enantioselectivity, especially for irreversible hydrolytic reactions, stems from the Michaelis-Menten theory and from the rate constants such that $E = (k_{cat}/K_m)_R/(k_{cat}/K_m)_S$ (Chen et al. 1982). In order to calculate the real enantioselectivity, we measured the specificity constants at pH 5.0 and 50°C for wild-type and mutated enzymes. For wild-type EST2 and AFEST, measurements were also done at 37°C.

Pure EST2 and AFEST and mutants partially purified by thermoprecipitation at 65°C for 20 min were utilized for these calculations. Initial velocity versus substrate concentration data were fitted to the Lineweaver-Burk transformation of the Michaelis-Menten equation by weighted linear least-squares analysis with a personal computer and GRAFIT version 3.0 software (Erithacus Software, Staines, UK). Assays were done in duplicate or triplicate, and the results reported for kinetic data are the mean of two independent experiments. Conventional determination of E through assays on racemic substrates, determination of enantiomeric excesses, and percentages of conversion could not be performed due to technical problems in the separation of (*R*)- and (*S*)-chloropropionic acids.

DNA from clones with altered enantioselectivity was extracted by conventional procedures (Sambrook et al. 1989) and submitted to automatic DNA-sequencing analysis (Primm, Milan, Italy).

Substrate specificity of Leu212Pro

The time course of the esterase-catalyzed hydrolysis of *p*-nitrophenyl (pNP) esters with acyl chain lengths varying from 2 to 16 carbon atoms was followed by monitoring of *p*-nitrophenoxide production at 405 nm in 1-cm-path-length cells with a DU 600 spectrophotometer (Beckman). Initial rates were calculated by linear least-squares analysis of time courses comprising less than 10% of the total turnover.

Assays were performed at 70°C in mixtures of 40 mM Na_2HPO_4/NaH_2PO_4, 0.09% (w/v) gum arabic, and 7.5% propan-2-ol (pH 7.1) containing pNP esters at different concentrations. Stock solutions of pNP esters were prepared by dissolving substrates in pure propan-2-ol.

Molecular modeling

Computer modeling was performed at the SWISS-MODEL web server (http://www.expasy.ch/swissmod), which employs the ProMod and Gromos 96 programs (Guex and Peitsch 1997; Guex et al. 1999) for comparative modeling and energy minimization, respectively. We first used the EST2 (PDB code: 1EVQ) and AFEST (PDB code: 1JJI) structures to obtain an EST2 model with methionines in place of selenomethionines and without the serine-bound hydroxyethylpiperazine ethanesulfonic acid (HEPES) inhibitor, and an AFEST model without the serine-bound HEPES inhibitor. Then, we produced models of the two mutants by using as references the wild-type models. Wild-type and mutant models were superimposed and differences analyzed. The models were evaluated by the "What If" program (Vriend 1990) and by exploiting the tools available in version 3.7b2 of the visualization program Swiss-PdbViewer (Glaxo Wellcome Experimental Research, Geneva). The average global displacement and the average local RMSD obtained after protein superposition were calculated with version 2K1 of the MOLMOL program (Koradi et al. 1996). In the global displacement, whole molecules are superimposed and deviations are calculated for one residue. For the local RMSD, three residues are superimposed and deviations are calculated for these three residues and referred to the middle residue.

Results and discussion

We recently overexpressed two homologous carboxylesterases, EST2 and AFEST, in *E. coli* and characterized the recombinant purified enzymes with respect to enantioselectivity toward a number of substrates of interest in the pheromones and pharmaceutical fields (Manco et al. 1998). The enzymes were very effective as catalysts, and significant conversions were achieved in short reaction periods with high substrate/enzyme ratios. However, substantial enantioselectivity was observed with EST2 and AFEST only in the resolution of compounds (±)-3-bromo-5-(hydroxymethyl)-Δ^2-isoxazoline and (±)-6-methyl-5-hepten-2-ol, respectively.

For EST2, the (*R*)-product was obtained with an 84% enantiomeric excess at 36% conversion. The enantiomeric ratio was 18. In the case of AFEST, the (*R*)-product was obtained with a 60% enantiomeric excess at 39% conversion. The enantiomeric ratio was 6 (Manco et al. 2000a).

To test the powerful technique of directed evolution on these enzymes and to improve their seemingly low selectivity, the two wild-type genes were mutated using error-prone PCR (epPCR). The substrate chosen for screening was *p*-nitrophenyl-2-chloropropionate because (*S*)-2-chloropropionic acid is an important intermediate in the synthesis of the herbicide mecoprop. Preliminary analyses indicated that the selectivity toward this substrate was low as well. (*R*)-*p*-Nitrophenyl-2-chloropropionate and the (*S*)-*p*-nitrophenyl-2-chloropropionate were prepared by starting from (*R*)- or (*S*)-2-chloropropionic acid and *p*-nitrophenol (see Materials and methods for details).

Random mutagenesis and screening for mutants with altered enantioselectivity

DNA fragments containing hypothetical mutations induced at a rate of two per 1,000 bp were generated by epPCR (see Materials and methods). Although we did not perform a systematic search for the number of mutations that were actually introduced, we can infer that mutations were introduced at this rate because we did not find any clones with more than two mutations, and we did find 1–2 silent mutations in some clones. Moreover, several of our clones were inactive in a filter assay with β-naphthylacetate (Manco et al. 2001; see below), suggesting that there were mutations in residues important for stability or activity. The analysis of these clones will be the object of future work.

DNA fragments were cloned into the respective expression vectors and transformed in Top 10 cells. Colonies from plates (about 5,000) were transferred with sterile toothpicks to 96-well microtiter plates containing 200 µl of LB medium supplemented with ampicillin and incubated overnight at 37°C. The following day, after transferring the cultures by sterile replicators to nitrocellulose filters and staining the colonies with substrate β-naphthylacetate according to a previously described procedure (Manco et al. 2001), only active clones (about 3,000) were selected and picked up for further screening.

Assays were performed on two 96-well microtiter plates containing the (*R*)- or (*S*)-*p*-nitrophenyl-2-chloropropionate, as reported in Materials and methods. Clones that were reproducibly found to have an altered V_S/V_R ratio (apparent E) were cultured in a small volume; the enzymes were partially purified by thermoprecipitation of *E. coli* proteins at 65°C for 20 min and centrifugation, and then they were reassayed spectrophotometrically at pH 5.0 to confirm that the V_S/V_R ratios were altered with respect to the wild-type enzymes. After the thermoprecipitation step, the activity was fully recovered. As reported in Table 1, the apparent E ratio measured for the EST2 mutant at pH 5 was 1.05 compared with a value for the wild-type enzyme of 1.43. In the case of the wild-type and mutant AFEST, the values obtained were 1.06 and 1.45, respectively. Therefore, the EST2 mutant apparently displayed an about 1.4-fold increased preference for the (*R*) over the (*S*) substrate, whereas in the case of AFEST the opposite relationship was observed between the wild-type and mutant enzyme. Because real enantioselectivity is defined as the ratio between specificity constants, e.g., $(k_{cat}/K_m)_S/(k_{cat}/K_m)_R$, we performed these kinetic measurements at pH 5.0 and 50°C (Table 1). The true E values were found to be consistently altered, with a 5.6-fold difference between the wild-type EST2 and its mutant and a 4.7-fold difference between wild-type AFEST and its mutant. However, the true E values were opposite, in both cases, to the values obtained from the spectrophotometric assays on microtiters. This behavior could be partly explained by the fact that the K_m values were similar for wild-type AFEST and EST2 for both substrates but substantially altered in the respective mutants. In particular, the K_m values were increased in the EST2 mutant and decreased in the AFEST mutant for both substrates (data not shown). Moreover, the plate assays were performed at room temperature and in the presence of Triton X-100 and gum arabic, which were not present when the measurements of the constants k_{cat} and K_m were performed.

Because these enzymes are thermophilic, it was of interest to ascertain if there was a dependence of enantioselectivity on temperature. In fact, some experimental and theoretical studies have indicated that enzyme enantioselectivity should increase at temperatures higher or lower than the racemic temperature (Phillips 1996) when activity is the same for both enantiomers. Measurements for wild-type EST2 and AFEST were therefore also done at 37°C. The values obtained were 0.87 and 1.41, respectively, which are not particularly different from the values obtained at 50°C.

Two mutations, Leu101Ile and Asp117Gly, were found in the AFEST variant, whereas a single mutation, Leu212Pro, was found in the EST2 variant. Leu101 and Asp117 are both conserved between EST2 and AFEST, whereas the Leu212 in EST2 is substituted by Ser in AFEST. The locations of the mutations in the AFEST and EST2 structures are highlighted in Figs. 2A and B, respectively.

Further attempts to obtain mutants with higher enantioselectivity in a second round of mutagenesis and screening were unsuccessful with both genes. About 1,000 clones were screened for each gene and for each cycle. In addition, a sat-

Table 1. Measurement of enantioselectivity (as the V_S/V_R ratio or E) for wild-type EST2 and AFEST and their respective mutants

	Wild-type EST2	Leu212Pro	Wild-type AFEST	Leu101Ile/ Asp117Gly
V_S/V_R ratio[a]	1.43 ± 0.10	1.05 ± 0.05	1.06 ± 0.05	1.45 ± 0.10
E[b]	1.17	6.6	1.55	0.33

[a] Activity on microtiter plates at room temperature in 34 mM citrate buffer, pH 5.0, containing 28% propan-2-ol, 0.06% Triton X-100, and 0.29% gum arabic. Data are means ± SD ($n = 5$)

[b] $(k_{cat}/K_m)_S/(k_{cat}/K_m)_R$ ratio. Spectrophotometric assays at 50°C for wild-type and mutated EST2 and AFEST; substrates dissolved in 40 mM citrate buffer, pH 5.0, 10% propan-2-ol. Data are means of two experiments (error < 15%)

uration mutagenesis approach was applied to EST2 to insert all possible residues at position 212, but, after analyzing about 300 clones, we obtained no clones with significantly altered enantioselectivity with respect to the starting mutant. A possible explanation for the latter results is that the substrate used has a complexity too low to be a good candidate for evolving the esterases being studied. Probably, mutations able to change substantially the enantioselectivity must directly or indirectly modify the immediate surroundings of the active site serine, and by doing so they might severely depress catalytic activity. Therefore, these clones could have been lost with the screening protocol adopted. This conclusion stems from a number of considerations. The first is that a mutation near the active site serine, namely Gly84Ser in EST2, reduces the catalytic activity by 95% (Manco et al. 1999). In contrast, mutations at the acyl-binding site, far away from the serine, increased the activity and changed substrate specificity (Manco et al. 2001). Second, we obtained the same low variation in enantioselectivity for both enzymes, which, in spite of just 40% identity, adopt the same reaction mechanism. Finally, in a similar study by Jaeger and coworkers (Liebeton et al. 2000) of a lipase from *Pseudomonas aeruginosa* PA01, the results were more impressive, but the substrate used had a side chain of ten carbon atoms. It is conceivable that the longer acyl chain allows for more constraints that can be modified by mutations without affecting catalytic activity. Alternatively, a much higher number of clones should be analyzed and perhaps a higher mutagenic rate, e.g., the simultaneous introduction of more mutations, should be adopted to succeed with this system.

Molecular modeling

We recently succeeded in resolving the EST2 3-D structure (De Simone et al. 2000). It was therefore of interest to analyze the effect of the Leu212Pro mutation in its structural context. Leu212 is located inside an α-helix, with the lateral chain projecting from the surface. Its main-chain O and N form two hydrogen bonds each with the surrounding residues, which stabilize the helix. Therefore, we did not expect that the substitution of this residue by a proline would modify extensively the structure. To verify the point, we produced a model of EST2 containing proline in place of leucine at position 212. We first generated a model of wild-type EST2. We then replaced the four selenomethionines with regular methionines and eliminated the HEPES molecule bound to the active site from the model (De Simone et al. 2000). In addition, in doing so, we subjected the wild-type enzyme to the same modeling procedure as the mutant. Finally, we modeled the mutated sequence on the EST2 model and compared the two molecules. When the wild-type and mutant models were superposed, the RMSD was 0.05 Å on a superposition of 1,232 backbone atoms. A number of residues in the mutant were found to have an RMSD higher than 0.05 Å compared with the wild type (Fig. 1A). Some of these residues were located near the active site (data not shown). Among these, the residue most deviating in terms of local RMSD, and the most proximal to the HEPES-bound inhibitor, was Met211 (Fig. 2B). We demonstrated in a previous paper (Manco et al. 2001) by site-directed mutagenesis that the residue at position 211 is involved in the catalytic activity and specificity of EST2. Thus, we conclude that Met211 is also important for enantioselectivity with respect to the selected substrate. The next residue, Pro212, i.e., the mutation introduced, was the most deviating residue in terms of global displacement (Fig. 1A), but the main-chain hydrogen bonds were not affected by the mutation, as expected. No particular tendency was observed for the most deviating residues to be located in any particular structures. Thr59 was located in strand β1 and Ala123 in helix α4; Gly208 and Met211 were located in α-helices, and Pro122, Asp141, and Leu176 in loops. However, Thr59, Ala123, Pro122, and Asp141 were found clustered in a substructure comprising strands β1, β2, and β4, against which helix α4 is packed. Some hydrogen bonds stabilizing this substructure and some others in the adjacent oxyanion loop, which is implicated in the stabilization of the transition state as well as in the formation of the acyl-binding site (De Simone et al. 2000), were altered in the mutant compared with the wild-type enzyme.

We recently succeeded also in resolving the AFEST 3-D structure (De Simone et al. 2001). A model was constructed for AFEST with the two above outlined mutations by using as a reference the AFEST structure (PDB code: 1JJI). Ile101 and Gly117 were located in the Asp99–Ser109 α-helix and the adjacent Ser111–Gly117 β-strand, respectively (Fig. 2A). These two structural elements show several interactions with each other and with residues of the oxyanion loop (His86–His98; black region in Fig. 2A).

We compared the wild-type and the mutant models and found a 0.03 RMSD on a superposition of 1,160 backbone atoms (Fig. 1B). Once more, the differences were localized to residues surrounding the putative acyl-binding site such as Gly87 and Ser219 or pertained to residues such as Ile95 and Gly87 that are inside the oxyanion loop (His86–His98), which, as mentioned above, is implicated in the stabilization of the transition state and also in the formation of the acyl-binding site (De Simone et al. 2000, 2001). Several other differences were located far away from the acyl-binding site, such as Met4, Ser27, Ile101 (the mutation introduced), Ser115, Ser152, Lys 153, Phe264, Gly265, and Arg279. No particular tendency was observed for the most deviating residues to be located in particular structures except for Ser115, which is located in the β-strand containing the Asp117Gly mutation and forms van der Waals interactions with residues of the oxyanion loop. In conclusion, mutations obtained for the two genes were positioned differently in the structures, but resulted in similar small modifications around the active and acyl-binding sites. Interestingly, all the substitutions found in the evolved lipase from *Pseudomonas aeruginosa* (Liebeton et al. 2000) were located far away from the stereocenter of the substrate, and one was suggested to affect the stability of the oxyanion hole loop, some residues of which are also involved in van der Waals interactions with residues in the acyl-binding pocket (Liebeton et al. 2000). No residues directly involved in enantiomer discrimination were, however, identified in the case of *P. aeruginosa*, although the difference in enan-

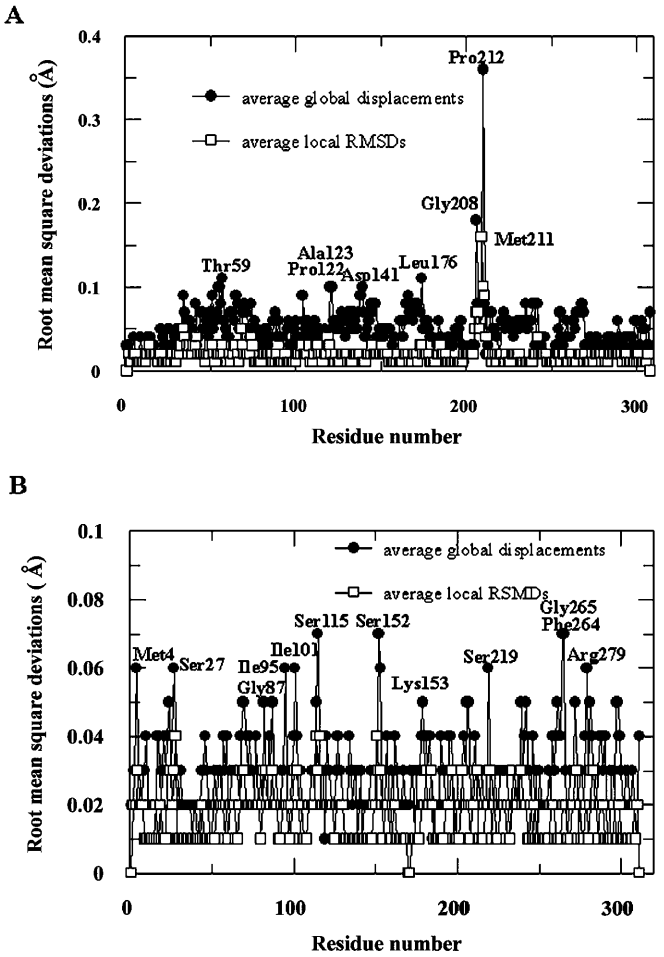

Fig. 1A,B. Average global displacement and local RMSDs for mutant Leu212Pro with respect to wild-type EST2 (**A**) and for double-mutant Leu101Ile/Asp117Gly with respect to wild-type AFEST (**B**). Residues are indicated that were two or more times the mean RMSD value. Calculations were performed with the MOLMOL program (Koradi et al. 1996)

tioselectivity reported by Liebeton et al. (2000) was clearly higher than that reported here. Quite interestingly, in the *Candida rugosa* lipase, too, Manetti et al. (2000), by a modeling study with docked substrates, did find conformational rearrangements of amino acid main chains of the oxyanion loop, among others, to be involved in the stereorecognition of (±)-2-(3-benzoylphenyl)propionate.

Further analysis of the purified EST2 mutant Leu212Pro

The EST2 mutant was purified to near homogeneity with a published procedure (Manco et al. 1998; data not shown), and the V_S/V_R ratio (the apparent enantioselectivity) was analyzed in the range 25°–55°C at 10°C intervals (data not shown). The V_S/V_R ratio was found to be substantially unchanged. Unfortunately, the temperature sensitivity of the substrate precluded any further analysis at higher temperatures to identify the racemic temperature of the reaction.

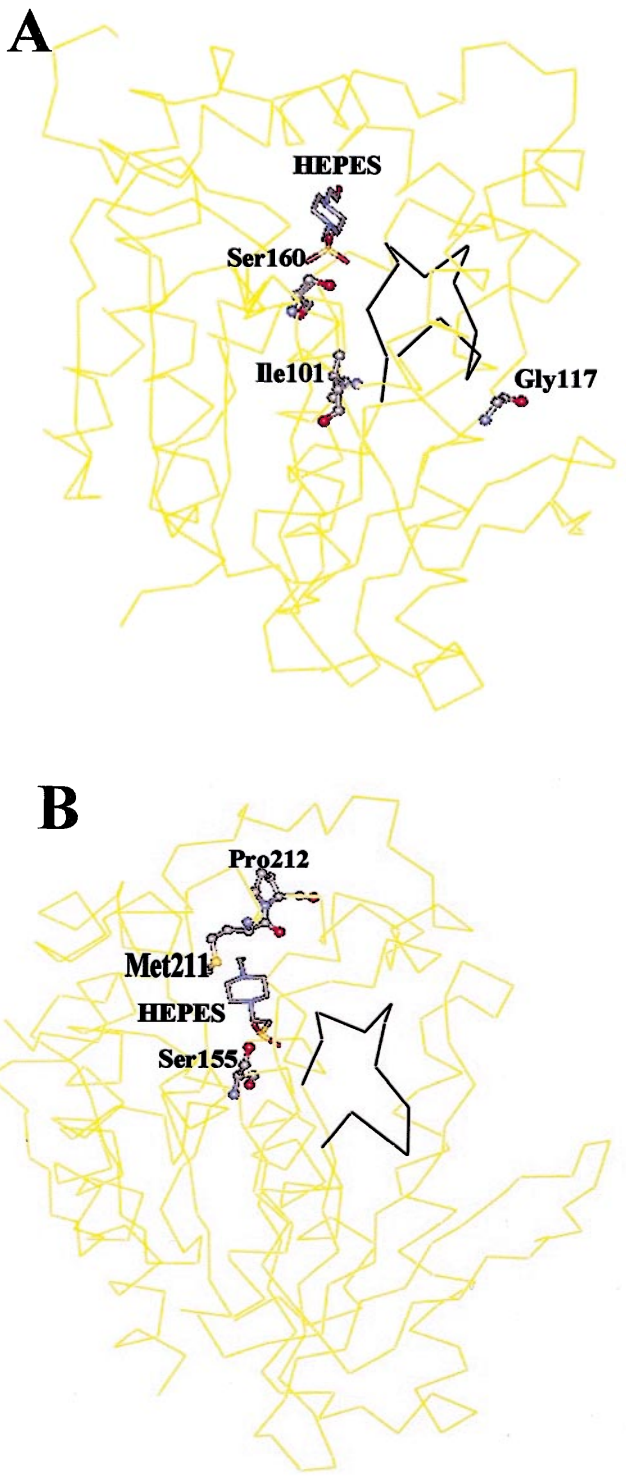

Fig. 2. **A** Cα trace of the Leu101Ile/Asp117Gly double-mutant model. Shown in *ball-and-stick* style are the two mutations and the active site, *Ser160*. The oxyanion loop is also shown in *black*. **B** Cα trace of the Leu212Pro mutant model. Pro212 and Met211 are drawn in *ball-and-stick* style. The oxyanion loop is shown in *black*. The hydroxyethylpiperazine ethanesulfonic acid (*HEPES*) molecules (*stick representation*) bound to the active site serines and lying in the acyl-binding pockets (De Simone et al. 2000, 2001) are superimposed on both models

The purified EST2 mutant was also compared with the wild-type with respect to substrate specificity with *p*NP esters with different acyl chain lengths. Quite interestingly, although the profile of substrate specificity of the mutant was similar to that of the wild-type enzyme for short chain *p*-nitrophenyl ester substrates (acyl chain lengths from two to eight carbon atoms), the activity toward substrates with 12–18 carbon atoms (C_{12}–C_{18}) was significantly reduced (data not shown). This result is in line with those of previous studies (Manco et al. 2001), indicating that the region where the Leu212Pro mutation is located is important for substrate specificity.

Conclusions

We have changed in a significant way the enantioselectivities of two thermostable esterases after a single round of random mutagenesis and screening. By modeling studies of the mutations in their structural context, only indirect effects, in terms of main chain movements, were observed for some residues around the active site. Work is in progress to change further the enantioselectivities and to study directly, by X-ray crystallography, the effects of mutations.

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References

Carrea G, Riva S (2000) Properties and synthetic applications of enzymes in organic solvents. Angew Chem Int Ed Engl 39:2226–2254

Chen CS, Fujimoto Y, Girdaukas G, Sih CJ (1982) Quantitative analysis of biochemical kinetic resolutions of enantiomers. J Am Chem Soc 104:7294–7299

De Simone G, Galdiero S, Manco G, Lang D, Rossi M, Pedone C (2000) A snapshot of a transition state analogue of a novel thermophilic esterase belonging to the subfamily of mammalian hormone-sensitive lipase. J Mol Biol 303:761–771

De Simone G, Menchise V, Manco G, Mandrich L, Sorrentino N, Lang D, Rossi M, Pedone C (2001) The crystal structure of a hyperthermophilic carboxylesterase from the archaeon *Archaeoglobus fulgidus*. J Mol Biol 314:507–518

Faber K, Ottolina G, Riva S (1993) Selectivity-enhancement of hydrolase reactions. Biocatalysis 8:91–132

Frederickson R (1999) Exploiting diversity. Nat Biotechnol 17:1150

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18:2714–2723

Guex N, Diemand A, Peitsch MC (1999) Protein modelling for all. Trends Biochem Sci 24:364–367

Henke E, Bornscheuer UT (1999) Directed evolution of an esterase from *Pseudomonas fluorescens*. Random mutagenesis by error-prone PCR or a mutator strain and identification of mutants showing enhanced enantioselectivity by a resorufin-based fluorescence assay. Biol Chem 380:1029–1033

Koradi R, Billeter M, Wüthrich K (1996) MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graphics 14:51–55

Liebeton K, Zonta A, Schimossek K, Nardini M, Lang D, Dijkstra BW, Reetz MT, Jaeger KE (2000) Directed evolution of an enantioselective lipase. Chem Biol 7:709–718

Manco G, Adinolfi E, Pisani FM, Ottolina G, Carrea G, Rossi M (1998) Overexpression and properties of a new thermophilic and thermostable esterase from *Bacillus acidocaldarius* with sequence similarity to hormone-sensitive lipase subfamily. Biochem J 332:203–212

Manco G, Febbraio F, Adinolfi E, Rossi M (1999) Homology modeling and active-site residues probing of the thermophilic *Alicyclobacillus acidocaldarius* esterase 2. Protein Sci 8:1789–1796

Manco G, Camardella L, Febbraio F, Adamo G, Carratore V, Rossi M (2000a) Homology modeling and identification of serine 160 as nucleophile of the active site in a thermostable carboxylesterase from the archaeon *Archaeoglobus fulgidus*. Protein Eng 13:197–200

Manco G, Giosuè E, D'Auria S, Herman P, Carrea G, Rossi M (2000b) Cloning, overexpression, and properties of a new thermophilic and thermostable esterase with sequence similarity to hormone-sensitive lipase subfamily from the archaeon *Archaeoglobus fulgidus*. Arch Biochem Biophys 373:182–192

Manco G, Mandrich L, Rossi M (2001) Residues at the active site of the esterase 2 from *Alicyclobacillus acidocaldarius* involved in substrate specificity and catalytic activity at high temperature. J Biol Chem 276:37482–37490

Manetti F, Mileto D, Corelli F, Soro S, Palocci C, Cernia E, D'Acquarica I, Lotti M, Alberghina L, Botta M (2000) Design and realization of a tailor-made enzyme to modify the molecular recognition of 2-arylpropionic esters by *Candida rugosa* lipase. Biochim Biophys Acta 1543:146–158

May O, Nguyen PT, Arnold FH (2000) Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. Nat Biotechnol 18:317–320

Petrounia IP, Arnold FH (2000) Designed evolution of enzymatic properties. Curr Opin Biotechnol 11:325–330

Phillips RS (1996) Temperature modulation of the stereochemistry of enzymatic catalysis: prospects of exploitation. Trends Biotechnol 14:13–16

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd Ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Vriend G (1990) WHAT IF: a molecular modeling and drug design program. J Mol Graphics 8:52–56