Homology modeling and active-site residues probing of the thermophilic *Alicyclobacillus acidocaldarius* esterase 2

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Abstract

The moderate thermophilic eubacterium *Alicyclobacillus* (formerly *Bacillus*) *acidocaldarius* expresses a thermostable carboxylesterase (esterase 2) belonging to the hormone-sensitive lipase (HSL)-like group of the esterase/lipase family. Based on secondary structures predictions and a secondary structure-driven multiple sequence alignment with remote homologous protein of known three-dimensional (3D) structure, we previously hypothesized for this enzyme the α/β -hydrolase fold typical of several lipases and esterases and identified Ser155, Asp252, and His282 as the putative members of the catalytic triad. In this paper we report the construction of a 3D model for this enzyme based on the structure of mouse acetylcholinesterase complexed with fasciculin. The model reveals the topological organization of the fold corroborating our predictions. As regarding the active-site residues, Ser155, Asp252, and His282 are located close to each other at hydrogen bond distances. Their catalytic role was here probed by biochemical and mutagenic studies. Moreover, on the basis of the secondary structure-driven multiple sequence alignment and the 3D structural model, a residue supposed important for catalysis, Gly84, was mutated to Ser. The activity of the mutated enzyme was drastically reduced. We propose that Gly84 is part of a putative “oxyanion hole” involved in the stabilization of the transition state similar to the C group of the esterase/lipase family.

Keywords: catalytic triad; homology modeling; oxyanion hole; site-directed mutagenesis; thermophilic esterase

Some studies (Krejei et al., 1991; Hemilä et al., 1994) have suggested that esterases, lipases, and cholinesterases belong to a large family of phylogenetically related proteins with representatives in the domains of eukarya and bacteria and include proteins lacking enzymatic activity. Three subfamilies have been identified. (1) The C group includes cholinesterases from vertebrate and invertebrate, lipases from fungi, a number of esterases, and some nonenzymatic proteins. (2) The L group includes lipases from vertebrate and bacteria, lipoprotein lipases, lecithin-cholesterol acyltransferases, and related nonenzymatic vitellogenins from flies. (3) The third group, H, was also named HSL by Hemilä et al. (1994). These authors reported the cloning and sequencing of a gene from *Alicyclobacillus acidocaldarius*, which encoded a protein of unknown function (ORF3) homologous to the HSL from human and

rat (Langin et al., 1993). In this group were also included the N-acetylphosphino-thricintripeptide deacetylase from *Streptomyces hygroscopicus* (Raibaud et al., 1991); the acetylhydrolase from *Streptomyces viridochromogenes* (Wohelleben et al., 1988); the lipase 2 from *Moraxella* TA144 (Feller et al., 1991a); an esterase from *Acinetobacter calcoaceticus* (Reddy et al., 1989); the esterase encoded by the ORF203 of *Escherichia coli* (Kanaya et al., 1998); Vsh5, a protein from the slime mold *Dictiostelium discoideum* (Singleton et al., 1989); the human liver arylacetamide deacetylase (Probst et al., 1994), and a recently identified *Archeoglobus fulgidus* esterase (Manco et al., 1998b; G. Manco, E. Giosue, G. Carrea, & M. Rossi, unpubl. results). Unfortunately, it is unclear at the moment whether the sequence similarities among these enzymes imply comparable biochemical properties. Moreover, three-dimensional (3D) structures are only available for the C and L groups.

Recently we reported on the purification of a new esterase (EST2) from *A. acidocaldarius* and demonstrated its identity with the aforementioned ORF3. On the basis of a secondary structure-driven multiple sequence alignment, we predicted for this enzyme the α/β -hydrolase topological fold common to several esterases and lipases and identified Ser155, Asp252, and His282 as the putative members of the catalytic triad (Manco et al., 1997).

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Abbreviations: CD, circular dichroism; DFP, di-isopropylphosphorofluoridate; EST2, esterase 2; HPLC, high-performance liquid chromatography; HSL, hormone-sensitive lipase; MAH, mouse acetylcholinesterase; PAGE, polyacrilamide gel electrophoresis; PDB, Protein Data Bank; PNP, p-nitrophenol; PTH, phenylthiohydantoin; RMSD, root-mean-square deviation; TFA, trifluoroacetic acid; UV, ultraviolet.

Fig. 1. Alignment of the structural template MAH and the EST2 sequence. Identical residues are in bold. In each block, top and bottom lines represent secondary structure elements detected in MAH and those predicted for EST2, respectively. Numbering for MAH is according to the PDB entry: 1MAH.

The gene was overexpressed in *E. coli*, purified and characterized to obtain further insights into the structure-function relationship of this interesting representative of the HSL group (Manco et al., 1998a). The enzyme has been crystallized and resolution of the 3D structure is currently under way (De Simone et al., 1999; G. De Simone, G. Manco, M. Rossi, & V. Parone, unpubl. results).

In the current absence of any 3D structure for the HSL group of enzymes, we decided to exploit a combined approach of site-directed mutagenesis and molecular modeling to investigate the structural features of the enzyme.

Results and discussion

The sequence identity in pairwise comparisons between EST2 and members of the C and L groups of the esterase/lipase family whose 3D structure is known is very low (about 30% or less) (data not shown). Nevertheless we hypothesized for EST2 the α/β -hydrolase fold on the basis of secondary structure predictions and a "threading" technique (Manco et al., 1997), the latter being a powerful means to identify remote relatives of proteins when the sequence identity is around 25%, a region of sequence identity that is frequently called the "twilight zone" (Clothia & Lesk, 1986; Sander & Schneider, 1991). The same conclusions were reached by Contreras et al. (1996) for the human HSL and allowed the construction of a partial homology modeling of the protein enzymatic domain. For our modeling purposes we generated a secondary structure-driven sequence alignment (Fig. 1) between EST2 and the mouse acetylcholinesterase (MAH), as previously reported (Manco et al., 1997) and submitted this alignment to the modeling procedure (see Materials and methods). Figure 2 shows the general folding pattern of the EST2 model obtained by using as a reference the 3D structure, solved at 3.2 Å (PDB entry: 1MAH) (Bourne et al., 1995), of MAH complexed with the inhibitor fasciculin. The superimposed backbone traces of EST2 and MAH (data not shown) displayed, by using a 2.75 Å cut-off distance, a 1.49 Å RMS deviation (RMSD) on 235 C α atoms. In a global superposition (305 C α atoms), the RMSD was 2.98 Å. Good values were also obtained in the superposition with the *Candida rugosa* lipase (CRL;

PDB entry: 1CRL; 273 C α atoms; RMS = 3.21 Å) and the *Geotrichum candidum* lipase (THG; PDB entry: 1THG; 267 C α atoms; RMS = 4.48 Å). The Ramachandran plot (data not shown) indicate that most (95%) of residues have ϕ and ψ angles in the core and allowed regions, except for some proline and glycine residues and some residues located in loop regions. As expected, Ser155 being the putative nucleophile, displayed anomalous ϕ and ψ angles. Most bond lengths, bond angles, and torsion angles were in the range of values expected for a naturally folded protein (data not

Fig. 2. Schematic representation of the structural model of EST2. The figure was generated with Insight II package. Strands labeled $\beta 1$ – $\beta 8$ pertain to the prototypic α/β -hydrolase fold whereas $\beta 0$, $\beta 2$, and $\beta 3$ represent strands outside the canonical α/β -hydrolase fold. Helices were labeled sequentially as $\alpha 1$ – $\alpha 10$.

shown). The schematic structural model shown in Figure 2 consists of two β -sheets: The central β -sheet is made up of nine β -strands ($\beta 0$ – $\beta 8$) of which $\beta 1$ – $\beta 8$ correspond to the canonical α/β fold (Ollis et al., 1992). Differences are represented by absence of $\beta 1$ ($\beta 1$, $\beta 2$, $\beta 3$, and $\beta 0$ indicate strands outside the canonical α/β fold in MAH) and the four α -helices after $\beta 7$, as well as the absence of the last two β -strands and the last α -helix ($\beta 9$, $\beta 10$ and α -10). The second strand of the central sheet corresponds to $\beta 1$ (Gly26–Phe32 in MAH) according to the accepted nomenclature (Ollis et al., 1992). β -strands 1–8 are twisted by about 90°.

The identified strands were: $\beta 0$ (Val6–Ile7); $\beta 1$ (Val10–Asp12); $\beta 2$ (Lys61–Tyr65); $\beta 3$ (Tyr74–Tyr80); $\beta 4$ (Val107–Val111); $\beta 5$ (Leu144–Val151); $\beta 6$ (Ala177–Phe178); $\beta 7$ (Pro244–Ala247); and $\beta 8$ (Val272–Phe275). In the case of human HSL, the N-terminal of the protein could not be modeled due to low sequence identity in this region with the templates MAH and CRL (Contreras et al., 1996). Our alignment based on GOR II prediction method for the latter region, resulted in an extra two-stranded β -sheet made up of β -2 (Asp4–Pro5) and β -3 (Lys23–His24), corresponding to similar elements in MAH, CRL, and THG. Note that $\beta 2$ and $\beta 0$ are undivided. As the identity in this region very low, the assignments are only tentative.

The “lid” or “flap” region is considerably shorter in EST2, in respect to THG and CRL, and is represented by the short helix Glu43–Ala46 corresponding to the helix Phe80–Trp86 in 1MAH. This finding is in line with the notion that EST2 is not a lipase (Manco et al., 1998a) even if the exact meaning of the term lipase is still a matter of debate (Verger, 1997).

According to the α/β -hydrolase fold (Ollis et al., 1992) and our previously made predictions (Manco et al., 1997), Ser155, Asp252, and His282 were located close to each other and connected by hydrogen bonds as in MAH (data not shown) thus representing a classical catalytic triad. The above results agree with our published biochemical data (Manco et al., 1998a) and with site-directed mutagenesis data reported for human HSL (Østerlund et al., 1997) and the *Moraxella* TA144 lipase (Feller et al., 1991b). The active-site serine is part of a conserved sequence, GX SXG, GDSAGG in the HSL group, which has been found in many other enzymes containing a catalytic triad (Brenner, 1988). In the model, the serine is located in a short turn between β -strand 5 and α -helix 5 in a strained conformation with backbone torsion angles: $\Phi = 80.4^\circ$ and $\Psi = -120^\circ$. This exposes the hydroxyl group to solvent and to the catalytic histidine. For steric reasons the supersecondary structure strand-turn-helix requires glycines at positions +2 and –2 with respect to the nucleophile Ser155. For the same reason, a residue with a short lateral chain, Ala156 in this case, is placed after the serine. The aspartic acid before the serine in the C group is substituted by glutamate and has been proposed to be involved in the coordination of water needed for substrate hydrolysis (Cygler et al., 1993).

To further test the role of Ser155 as the nucleophilic element of the triad, we tried a biochemical approach. The pure enzyme was labeled as previously described (Manco et al., 1998a) with [^3H]-diisopropylphosphorofluoridate (DFP), which totally inhibits activity of serine-type enzymes by phosphorylating irreversibly the essential nucleophilic residue. It was made free of unreacted inhibitor by gel filtration on a Sephadex G-25 column, and the labeled protein was finally trypsin digested. A sample of the untreated EST2 was preliminarily digested in the same way to set up the proteolysis and separations conditions (Fig. 3B). Proteolytic fragments were separated onto a reverse-phase C18 column (Fig. 3A) with a gradient

Fig. 3. Isolation of a [^3H]DFP-labeled peptide by reversed-phase HPLC chromatography. **A:** Pure EST2 (250 μg) was [^3H]DFP-labeled and digested with trypsin. Fragments were separated with the indicated acetonitrile gradient in 0.1% TFA. Almost all radioactivity (90%) co-migrated with the peak eluted at 31 min (indicated by an arrow). **B:** Unlabeled pure EST2 (150 μg) treated as above to set up proteolysis and separation conditions. **C:** Radioactive peak from run shown in A, reloaded on the same column and eluted with the indicated acetonitrile gradient in 0.1% TFA. The peak (indicated by an arrow) was subjected to Edman degradation.

of acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA). A major peak of radioactivity comigrating with the peak at 31 min, eluted at 40% (v/v) of acetonitrile. This material was reloaded on the same column and eluted with a shallower acetonitrile gradient from 35–55% (v/v) in 0.1% TFA (Fig. 3C). Some smaller components were separated from a prominent peak that was lyophilized and subjected to Edman degradation. As shown in Table 1, three trypsin fragments were identified based on the relative amounts of recovered phenylthiohydantoin (PTH) amino acids. Interestingly, one of these fragments contained the signature sequence GDSAGG. It was identified as the tryptic fragment 149–169. The other two fragments were 121–138 and 216–255. At the seventh cycle, where Ser155 was expected to be eluted, a very low signal for serine residue could be detected. It is worth noting that at the sixth cycle a serine was detected due to peptide 216–255 (Ser221). Thus the

Table 1. Peptides from trypsinized [^3H]DFP-labeled EST2^a

Cycle	Peptide 121–138	Peptide 149–169	Peptide 216–255
1	F(352)	I(25)	D(17)
2	P(277)	A(49)	Q(53)
3	A(372)	V(28)	Y(33)
4	A(432)	G(108)	L(59)
5	V(321)	G(113)	N(27)
6	E(149)	D(19)	S(22)
7	D(109)	S(10)	L(46)
8	A(304) ^b	A(304) ^b	E(30)
9	Y(191)	G(108)	E(27)
10	D(104)	G(106)	L(41)
11	A(227)	N(19)	T(15)
12	L(187) ^b	L(187) ^b	H(9)
13	Q(141)	A(58)	P(23)
14	W(193) ^b	A(43)	W(193) ^b
15	I(2)	V(23)	F(6)
16	A(19)	T(8)	S(8)
17	E(8)	S(9)	P(8)
	R	I	-22aa-
		L	R
		A	
		K	

^aNumber in parentheses indicate the picomoles of recovered PTH-amino acid.

^bPositions where the same residue is present in two sequences so that the recovered picomoles of PTH-amino acid referred to both residues.

10 pmol of serine found at the seventh cycle could be partly due to a leftover of the previous cycle. Peptides 121–138 and 216–255 are likely to be chymotryptic fragments. In fact at the 15th cycle, a dramatic reduction of signal for Ile135 and Phe230 was observed. The second serine (Ser165) of peptide 149–169 was detected, although at very low level, at the last cycle. These results strongly suggest that Ser155, embedded in the signature sequence GDSAGG, was labeled with [^3H]DFP and therefore was not eluted at the expected position. Thus, Ser155 is very likely to be the nucleophile of the catalytic triad.

In the HSL group the putative active-site His is contained in the conserved sequence HGF (Hemilä et al., 1994; Manco et al., 1997, 1998b). In our model this sequence is embedded in a turn between strand $\beta 8$ and α -helix $\alpha 8$. Regarding the acid, no consensus sequence could be found in the HSL group (Hemilä et al., 1994; Manco et al., 1997, 1998b).

To further test the role of Asp252 and His282 as members of the triad, we mutagenized these residues to Asn and Gln, respectively. The proteins were expressed in *E. coli*, although at a lower level compared with the wild-type (data not shown), as verified by Western blot analysis with the specific EST2 antiserum (Fig. 4), especially for the Asp252Asn mutant, which consequently could not be purified. No activity was detected in all the crude extracts with the standard assay. We purified the His282Gln mutant with a previously published procedure (Manco et al., 1998a) by monitoring the enzyme by SDS/PAGE and Western blot analysis. The pure enzyme proved to be correctly folded because (1) it had the same behavior of wild-type in the purification procedure, which includes three thermoprecipitation steps; (2) it had the same far-UV CD spectra of wild-type (Fig. 5A) at 25 °C as well as the same structural thermostability as inferred from the far-UV CD measure-

Fig. 4. Western blot analysis of mutants His282Gln, Gly84Ser, and Asp252Asn. A 50 μg portion of crude extracts from mutants His282Gln (lane b), Gly84Ser (lane c), and Asp252Asn (lane d) were fractionated by SDS/PAGE and subjected to the Western blot procedure. Pure EST2 (1 μg) was run as control (lane a).

ments at temperatures from 50–95 °C (Fig. 5B). Residual activity was detected with the purified His282Gln mutant, corresponding to 0.6% of wild-type activity. Thus we concluded that the loss of activity was ascribed to the introduced mutation and not to gross modification of the enzyme structure. Unfortunately, the same conclusion, in the absence of the pure enzyme, could not be made for the Asp252Asn mutant. The next question was if the loss of the Asp252 and His282 blocked the turnover of the substrate at the active site, or only the serine reactivity was impaired. To clarify this point crude extracts of *E. coli* expressing wild-type EST2 at different levels (Manco et al., 1998a) or the Asp252Asn or His282Gln mutated proteins were labeled with [^3H]DFP. As shown in Figure 6 the labeling of wild-type EST2 paralleled the expression of the protein as previously demonstrated by Western blot (Manco et al., 1998a), while no labeling occurred with the His282Gln mutant. The same result was obtained with the Asp252Asn mutant (data not shown). A faint band, however, was observed for the His282Gln mutant after longer film exposure. This result could indicate that most of the serine reactivity is due to the so-called “charge-relay” system, e.g., the capability of the Asp252 side chain to stabilize the positive charge generated on the histidine ring upon the proton transfer from the hydroxyl group. The residual activity of mutant His282Gln could be ascribed instead to the intrinsic reactivity of the serine hydroxyl group, owing to its topological position.

To further address the problem of the mechanism of action of EST2 we mutagenized Gly84, a residue supposed important for catalysis on the basis of sequence alignments and the 3D structural model. In fact, this residue belongs to the conserved sequence H/FGGG/A and represents together with the backbone nitrogen of the residue that follows the nucleophile, the so-called “oxyanion hole” (Sussman et al., 1991). In the C group of the esterase/lipase family, the former glycine stabilize the transition state with two hydrogen bonds from the amide of backbone. In several enzymes of the esterase/lipase family, there is a serine in this position (Cygler et al., 1993). In addition, the analysis of the transition state from the 3D structures of the *Rhizomucor miehei* lipase (Brzo-

Fig. 5. **A:** Far-UV CD analysis of His282Gln and Gly84Ser mutants. CD measurements of pure EST2 (continue line), pure His282Gln (dashed line), and Gly84Ser (dotted line) mutated proteins were performed at 25 °C at a protein concentration of 0.1 mg/mL in 40 mM phosphate buffer pH 7.4. **B:** Spectra were also recorded ranging from 50–95 °C. Only the ellipticity change at $\lambda = 222$ nm as function of temperature was reported.

zowski et al., 1992; Derewenda et al., 1992) and the *Fusarium solani* cutinase (Martinez et al., 1992) has demonstrated that serine in this position stabilizes the oxyanion with a third hydrogen bond from the hydroxyl group. A serine in this position is present also in lipases from *Humicola lanuginosa*, *Rhizopus delmar*, and *Penicillium camembertii* (Derewenda et al., 1994). For the above reasons, Gly84 was mutated to Ser.

Fig. 6. Fluorography analysis of [^3H]DFP-labeled wild-type EST2 expressed in Top 10 and BL21(DE3) *E. coli* cells and of [^3H]DFP-labeled His282Gln mutant. A 50 μg portion of total protein from Top 10 *E. coli* cells harboring plasmid pKTH2000 (Manco et al., 1998a) (lane b), from noninduced (lane c) or isopropyl-thiogalattopyranoside-induced (lane d) BL21(DE3) *E. coli* cells harboring plasmid pT7-SCII-GMI (Manco et al., 1998a), or from mutant His282Gln (lane e), were fractionated onto 12.5% SDS/PAGE, incubated with [^3H]DFP and treated for fluorography. Five micrograms of purified EST2 (lane a) treated in the same way was run as control. Mobility of molecular-mass markers are indicated on the left.

The mutated enzyme Gly84Ser was expressed (Fig. 4, lane c) purified and compared with the wild-type. No differences were observed at the level of secondary structures as measured by CD and shown in Figure 5A or in the structural thermostability (Fig. 5B), indicating no gross change in the structure but the specific activity was reduced 90-fold in the standard assay, being 5,300 U/mg for wild-type EST2 and 58 U/mg for mutant Gly84Ser. Finally the enzyme thermostability for wild-type EST2 and mutant Gly84Ser was evaluated by measuring the residual activity as described (Manco et al., 1998a). After incubation at 70 °C for 90 min, no loss of enzyme activity was observed for both enzymes. Thus we propose that Gly84 is part of a putative “oxyanion hole” involved in the stabilization of the transition state similar to what happens in the C group of the esterase/lipase family. A similar role for the corresponding glycine in HSL (Gly353) has been proposed on the basis of homology modeling (Contreras et al., 1996). However, to our knowledge this is the first mutant of a residue contributing to a putative “oxyanion hole” in the HSL group of the esterase/lipase family. A more extensive kinetic and structural analysis of the mutant is currently under way.

Materials and methods

Materials

All chemicals were reagent grade. p-Nitrophenyl(PNP) exanoate was purchased from Sigma Chemical Co. (St. Louis, Missouri). Molecular mass markers for SDS/PAGE and the kit for the T7-Sequencing™ were obtained from Pharmacia (Uppsala, Sweden). Trypsin was from Boehringer Mannheim (Mannheim, Germany). [^3H]Di-isopropylphosphofluoridate (DFP) (6 Ci/

mmol) and ^{35}S -dATP for sequencing were from Amersham Pharmacia Biotech (Uppsala, Sweden). Oligonucleotides were obtained from Primm srl (Milan, Italy). DNA restriction and modification enzymes were from Promega (Madison, Wisconsin).

Strains and plasmids

E. coli Top 10 (Invitrogen, Carlsbad, California) was used as host for cloning, whereas *E. coli* BL21 (DE3) harbored the recombinant plasmid for gene expression. The construct utilized for the expression (PT7-SCII-MG1) was described previously (Manco et al., 1998a).

Homology modeling

Computer modeling was performed on a Silicon Graphics O2 workstation using the software packages Homology and Discover in Insight II (Biosym/MSI, San Diego, California).

The sequence alignment between EST2 and MAH (PDB entry: 1MAH) (Bourne et al., 1995) was based on a published multiple sequence alignment which in turn relied on secondary structures correspondences (Manco et al., 1997) and confirmed completely with the program GORII implemented in the homology module of the Insight II package except for the N-terminal (see Results and discussion). Structurally conserved regions (SCR) were essentially based on the central structural elements found in the MAH structure.

Using the homology module of the Insight II package, the atomic coordinates of the SCR backbone and identical amino acids were copied from acetylcholinesterase, while mutated side chains were oriented similarly to the corresponding side chains in acetylcholinesterase.

The loops connecting the SCRs were modeled with the function "search loop," which searches a set of selected PDB structures for loops that best fit the given structural environment.

Side-chain conformations were manually refined and the most severe steric overlaps removed. Several cycles of constrained energy minimization regularized the structure and geometrical parameters.

The model was validated by both the PROCHECK program (Laskowski et al., 1993) of Insight II and by using the program WHAT IF (available free on the Internet—Web site: <http://biotech.ebi.ac.uk:8400/>).

Site-directed mutagenesis

Standard molecular cloning techniques were utilized (Sambrook et al., 1989). The mutants presented in this study were prepared by the overlap extension method (Ho et al., 1989) with the polymerase chain reaction (PCR). The plasmid PT7-SCII-MG1 (Manco et al., 1998a) was used as the template for amplification reactions carried out with the Expand high-fidelity PCR system (Boehringer Mannheim), using two end primers and two complementary mutagenic primers for each site-specific mutation. The sequences of the oligonucleotides used were as follows (mismatched sites for site-directed mutagenesis are underlined): Cter(−), 5′-TTG GATCCGCCTTTTGGTCAGG-3′; His282(−), 5′-CTGTGCGA ATCCCTGGATCAGATCTTCGAAG-3′; His282(+), 5′-GAA GATCTGATCCAGGATTCGCAC-3′; ASP252(−), 5′-CGC GCAGCGGATGTACTGCGCCG-3′; ASP252(+), 5′-CGGC GCAGTACAATCCGCTGCGCG-3′; Gly84(+), 5′-TATCACG

GCGGCAGTTGGGTCGTCGG-3′; Gly84(−), 5′-GCGTCTCG AGGTCTCCGACGACCAACTGCCGCCG-3′; Nter(+), 5′-G GCGACCCATATGCCGCTCGATCCC-3′. Each amplification reaction was performed with 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The final amplified products were cut appropriately with restriction enzymes, purified with an agarose gel-extraction kit (Quiaex II, M-Medical) and then ligated into PT77-SCII-MG1 linearized with the same restriction enzymes. The cloned DNA fragments were entirely sequenced to confirm the presence of the mutation and to rule out the possibility that replication errors were introduced during amplification.

Expression and purification of mutants His282Gln and Gly84Ser

Expression and purification of mutants and of recombinant wild-type enzyme were performed as described (Manco et al., 1998a). Purity was more than 95% as checked by SDS/PAGE.

Electrophoreses

Electrophoretic runs were performed with a Bio-Rad Mini-Protein II cell unit, at room temperature. SDS/PAGE was performed essentially as described by Laemmli (1970). Gels (12.5% polyacrylamide) were stained with Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany). Molecular mass markers (Pharmacia, Uppsala, Sweden) were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa). The molecular mass of the denatured enzyme was obtained by interpolation on a plot of log of molecular mass against relative migrations (R_f values).

Western-blot analysis

After the electrophoretic runs, SDS-gels were transferred onto a nitro-cellulose sheet in 0.375 M Tris/glycine buffer pH 8.3, containing 20% methanol, using the Bio-Rad Trans-Blot apparatus. After 1 h at 100 V, the nitro-cellulose sheet was removed, soaked in 20 mM Tris/HCl buffer pH 7.5 containing 3% bovine serum albumin (blocking buffer) and incubated for 1 h with the EST2 antiserum diluted (1:1,000) in blocking buffer. Thereafter, the membrane was washed three times for 15 min with 20 mM Tris/HCl buffer pH 7.5, containing 0.05% Tween 20, 0.5 M LiCl (washing buffer). The filter was incubated for 1 h with goat antirabbit conjugated with peroxidase (Sigma, St. Louis, Missouri) diluted (1:2,000) in blocking buffer. The filter was washed as described and developed by incubation in 0.1 M Tris/HCl buffer, pH 7.6, containing 3,3′-diaminobenzidine (0.3 mg/mL) and H_2O_2 (0.01%).

Protein labeling with [^3H]DFP and fluorography

Samples of 5–50 μg of protein were labeled by incubation with 2 μCi of [^3H]DFP in 25 μL of 50 mM phosphate buffer pH 7, 150 mM NaCl, at room temperature for 1 h. After the reaction, the unreacted inhibitor was separated from protein by gel filtration through Sephadex G-25. The recovered material was opportunely concentrated, the radioactivity measured with a Packard Tri-Carb 300 liquid scintillation counter, and aliquots corresponding to 1×10^3 and 1×10^4 cpm were loaded onto SDS/PAGE. After run

gels were treated for fluorography as described (Waterborg & Matthews, 1984).

Enzyme assays

The time course of the esterase-catalyzed hydrolysis of PNP esters was followed by monitoring the production of p-nitrophenoxide at 405 nm in 1 cm pathlength cells with a double-beam Varian Cary 1E ultraviolet (UV)-visible spectrophotometer equipped with a temperature controller (Varian, Palo Alto, California) based on Peltiers heat-exchange devices. Initial rates were calculated by linear least-squares analysis of time courses comprising less than 10% of the total substrate turnover. The standard assay contained 0.2 mM PNP exanoate in 40 mM Na₂HPO₄/NaH₂PO₄/0.09% (w/v) arabic gum/0.36% (v/v) Triton X-100/2% (v/v) propan-2-ol (pH 7.1) at 70 °C. Stock solutions of PNP esters were prepared by dissolving substrates in water, at their maximum of solubility (for kinetic measurements) or in propan-2-ol (for routine assays). The background hydrolysis of the substrate was deducted by using a reference sample of identical composition to the incubation mixture, except that esterase was omitted.

One unit of enzymatic activity was defined as the amount of protein releasing 1 µmol of p-nitrophenoxide/min from PNP ester at the indicated temperature. The pH was always adjusted for the appropriate working temperature.

The protein concentration measured with the Bio-Rad Protein Assay system, with BSA (Fraction V) as a standard, was found to be underestimated by 12% by amino acid composition analysis. Measures were corrected accordingly.

EST2 labeling and trypsin proteolysis

Pure esterase (25 nm in 0.5 mL of 20 mM Tris/HCl, pH 8) was titrated by addition of 150 µL of [³H]DFP (6 Ci/mmol; 167 pmol/µL; 4.6 × 10⁵ cpm/µL) and incubation at 37 °C. Aliquots were withdrawn at times from the reaction mixture, and the residual activity was assayed by the standard assay. After complete (98%) inactivation, the enzyme was separated from unreacted [³H]DFP by gel filtration through a column (20 × 0.5 cm) of Sephadex G-25, equilibrated and eluted with 40 mM phosphate buffer, pH 7. Protein eluted at the void volume was assayed by Bio-Rad protein assay and the co-eluted radioactivity was measured by using a Packard Tri-Carb 300 liquid scintillation counter. Fractions with the higher specific radioactivity were pooled, concentrated with a Centricon 10 apparatus, and subjected to trypsin proteolysis in 20 mM Tris/HCl buffer pH 8.0, containing 1 mM CaCl₂, for 14 h at 37 °C with a trypsin to protein ratio of 1:10. A similar amount of not-labeled EST2 was subjected to the same digestion as a control. Samples were made 0.1% (v/v) in TFA and loaded onto a reversed-phase HPLC C18 column, equilibrated in 0.1% TFA and eluted with an acetonitrile gradient. The eluted fragments were detected by UV absorption at 220 and 280 nm and aliquots were assayed for radioactivity as described above.

N-terminal amino acid sequence analysis

N-terminal amino acid sequence analysis was performed by automated Edman degradation with an Applied Biosystems (Foster City, California) gas-phase sequencer (model 477 A) equipped with an on line 120 A phenylthiohydantoin analyzer.

CD analysis

Far-UV (190–240 nm) CD measurements were performed in a spectropolarimeter model J-710 (Jasco, Tokyo, Japan) at 25 °C under nitrogen flow. A cuvette (Helma, Jamaica, New York) of 0.1 cm pathlength was used. Photomultiplier absorption did not exceed 600 V in the spectral region measured. A spectral acquisition spacing of 0.2 nm (1.0 nm bandwidth) was used. Each spectrum was averaged five times and smoothed with Spectropolarimeter System Software Ver. 1 (Jasco). The percentages of secondary structures were measured according to Yang (1986). To compare the structural thermostability of wild-type and mutant enzymes, spectral acquisition was achieved in the temperature range of 50–95 °C at 5 °C spacing. Moreover, the ellipticity reading at λ = 222 nm was continuously monitored as the temperature was raised.

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