# Identification of essential aspartic acid and histidine residues of hormone-sensitive lipase: apparent residues of the catalytic triad

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**Abstract** **It is expected that hormone-sensitive lipase (HSL), like most other lipases and esterases, adopts an α/β-hydrolase fold and has a catalytic triad of serine, aspartic or glutamic acid, and histidine. Recently, we have published a three-dimensional model for the C-terminal catalytic domain of HSL, having an α/β-hydrolase fold and with Ser-423[1], Asp-703 and His-733 in the catalytic triad (Contreras et al. (1996) *J. Biol. Chem.* 271, 31426–31430). It has been shown that Ser-423, situated in the motif GXSXG, is essential for catalysis (Holm et al. (1994) *FEBS Lett.* 344, 234–238). The suggested aspartic acid and histidine were here probed by site-directed mutagenesis. Mutants of residues Asp-703 and His-733 are devoid of both lipase and esterase activity, which is not the case for mutants of other tested aspartic acid and histidine residues. Thus, the presented data support the three-dimensional model structure with Asp-703 and His-733 as part of the triad.**



*Key words:* Lipase; Esterase; Site-directed mutagenesis; Active site; Catalytic triad

## 1. Introduction

Hormone-sensitive lipase (HSL) is a key enzyme in lipid metabolism and overall energy homeostasis, since it catalyzes the rate-limiting step in mobilization of triglycerides in adipose tissue (for reviews see [1–3]). HSL is under hormonal and neuronal control, involving activation through phosphorylation by the cAMP-dependent protein kinase [4,5]. The rat HSL cDNA has been cloned [6], sequenced [6,7] and expressed in COS cells [8] as well as in insect cells [9]. The latter has enabled purification of milligram amounts of highly pure HSL for studies of its structure–function relationships.

Common features of lipases and several esterases are that they adopt the so-called α/β-hydrolase fold [10] and perform catalysis using a catalytic triad consisting of a serine, aspartic or glutamic acid, and histidine (for reviews see [11,12]). Sequence alignment of HSL and *Moraxella* TA144 lipase 2, together with limited proteolysis and denaturation studies performed with recombinant HSL, suggested that HSL is formed by at least two structural domains [9]. Of these, the C-terminal domain was expected to have the α/β-hydrolase fold and accommodate the hydrolytic capacity [9]. Further analysis of HSL sequence and prediction of secondary structure elements revealed that the C-terminal domain is evolutionary related to enzymes of the esterase B family [13]. By comparing the predicted secondary structure elements with those of lipases and esterases of known structure from the esterase B family (*Geotrichum candidum* lipase, *Candida rugosa* lipase and *Torpedo californica* acetylcholine esterase), we have very recently built a three-dimensional model for the catalytic core of HSL [13]. One direct consequence of the structural model was the proposal of a complete catalytic triad: Ser-423[1], Asp-703 and His-733. Ser-423 is situated in a GXSXG motif, which is conserved around the active site serine in a large number of lipases and esterases. This residue has previously been probed by site-directed mutagenesis, and data from these analyses clearly identified Ser-423 as essential for both lipase and esterase activity of HSL [7]. Alignment of HSL and several other lipases and esterases including *Moraxella* TA144 lipase 2 [9,14], clearly support that the active site histidine is His-733, although no experimental data has been presented. In addition to the active site histidine and aspartic acid proposed by the model, there are alternatives that should be considered. A suggested motif GXXXXDG, for the active site aspartic acid of some lipases [11], is found at Asp-445 in HSL. Furthermore, it has also been suggested that a 17.6 kDa peptide, spanning amino acids 333 to 499, is a catalytically active core in HSL [15]. The only conserved histidine in this peptide, after the active site serine, is His-493 which consequently should be the active site histidine.

In the present work, the different candidate aspartic acid and histidine residues of the catalytic triad in HSL were probed by site-directed mutagenesis in order to provide experimental evidence on components of the active site. The validity of our structural model for the catalytic domain was further tested by this approach.

## 2. Materials and methods

### 2.1. Site-directed mutagenesis

Rat HSL cDNA, in the eukaryotic expression vector pSVL (Pharmacia) [8], was subjected to site-directed mutagenesis at codons for conserved aspartic acid and histidine residues. The mutations were introduced using the overlap extension method [16], as previously described [7]. High-fidelity Vent DNA polymerase (New England Biolabs) was used in order to minimize the occurrences of undesired mutations. A cassette approach [17] was used to replace fragments of the wild-type HSL cDNA by equivalent PCR-generated fragments containing the desired mutation.

### 2.2. DNA sequencing

The identity of each mutant and the absence of possible undesired mutations was confirmed by PCR-based automated DNA sequencing, using Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems) and a model 373A DNA sequencer (Applied Biosystems), according to instructions by the manufacturer.

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*Abbreviations:* HSL, hormone-sensitive lipase; MOME, 1(3)-monooleoyl-2-*O*-monooleyl glycerol; PNPB, *p*-nitrophenyl butyrate

[1]Residue numbers in this paper refer to the rat HSL amino acid sequence.


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*2.3. Transfection and expression of HSL cDNA*

Transfection and expression of wild-type and mutant rat HSL cDNAs in COS cells was accomplished by the lipofectin method as previously described [8]. For each construct, 5 μg of DNA was used to transfect 80% confluent COS cells in 60 mm plates. Transfections were performed in triplicate and at least two independent experiments were carried out for each construct. Cells were harvested 72 h after transfection. The cells were rinsed with phosphate-buffered saline, disrupted by a freeze/thawing cycle, and resuspended in 600 μl of 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 20 μg/ml leupeptin, 2 μg/ml antipain and 1 μg/ml pepstatin. Cell debris was removed by centrifugation at $12000 \times g$ for 2 min, and the supernatant was analyzed for protein concentration, lipase and esterase activity and HSL mass.

*2.4. Analysis of mutants*

Total protein in the supernatants was measured by a modified Lowry method [18]. Lipase activity was measured, by hydrolysis of 1(3)-mono-[$^3$H]oleoyl-2-*O*-monooleyl glycerol (MOME), a diglyceride ether analog, in a phospholipid stabilized emulsion, and esterase activity by hydrolysis of 0.5 mM *p*-nitrophenyl butyrate (PNPB) as described [9]. Background activities in the COS cells were determined from cells transfected with pSVL alone. All the analyses were done in triplicate.

*2.5. Western blot analysis*

The HSL mass was estimated by Western blot analysis. Briefly, 30 μg of total protein (15–20 μl) was subjected to SDS-PAGE using the Tris-tricine buffer system [19] and blotted onto nitrocellulose membranes (Hybond-C extra, Amersham) using standard procedures. Membranes were blocked for at least 2 h in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl supplemented with 5% defatted milk powder. All following washes were done 3 times, for 5 min each, in the same buffer with 2.5% milk powder and 0.25% Tween-20 (Bio-Rad). After blocking, membranes were washed and incubated with polyclonal chicken anti-rat HSL IgG for 2 h, followed by washing and incubation for 1 h with rabbit anti-chicken antibody IgG. After a new wash, membranes were incubated with $^{125}$I-labeled goat anti-rabbit antibody IgG for 1 h, washed and dried. Membranes were analyzed by digital imaging using a Fujix Bas 2000 Imager (Fuji). Electrophoresis and blotting were performed with different amounts of recombinant rat HSL in separate lanes in order to verify the linearity of the method for HSL.

## 3. Results and discussion

In order to probe the different candidate residues as to the active site aspartic acid and histidine in HSL, and to obtain experimental data to support our model for the catalytic core of this enzyme [13], residues Asp-445, Asp-703, His-493 and His-733 in rat HSL were subjected to site-directed mutagenesis. As further control residues, the conserved Asp-517 and His-696 were also mutated. Fig. 1 shows a schematic representation of the structural model for the catalytic core of HSL. The residues that were mutated in this work are shown in ball-and-stick representation, with the exception of His-493 and Asp-517. These two residues are located outside the region modeled, in an external module located between strands β6 and β7 [13]. Aspartic acid residues were mutated to asparagine and/or glutamic acid and histidines to serine and/or alanine in order to minimize the disturbance of protein structure [20]. Wild-type and mutant HSL proteins were expressed individually in COS cells. The activities of wild-type and HSL mutants were analyzed in cell homogenate supernatants as described under Section 2. Fig. 2 shows the lipase (A) and esterase (B) activities of COS cell supernatants containing wild-type or mutant HSL. Mutants of His-733 lack any detectable lipase or esterase activity. Since both lipase and esterase activities are affected, it is clear that the mutations disturb the hydrolytic capacity rather than the interaction of HSL with the lipid substrate interface. Mutating Asp-703 to asparagine also resulted in a complete inactivation of the enzyme. However, when the conservative mutation Asp-703-Glu was analyzed, the activity was dramatically reduced, but the possibility of some residual activity could not be ruled out ($P = 0.013$ vs. pSVL; Fig. 2). It is, however, not unusual that conservative substitution of the acidic residue of the triad does not completely inactivate lipases. This has been observed with both pancreatic lipase [21,22] and *G. candidum* lipase [23]. Mutants of Asp-445, Asp-517, His-493 and His-696 are only mildly affected in their activities (Fig. 2). Indeed, the Asp-517-Asn mutant is more active than wild-type HSL, especially with respect to esterase activity. When background activity is subtracted, Asp-517-Asn has approximately 1.7 times the esterase activity of wild-type HSL. The activity data clearly support that it is Asp-703 and His-733 that are important for catalysis and, therefore, possibly form part of the catalytic triad. In contrast, both alternative residues (Asp-445 and His-493) are largely unaffected by mutagenesis. The fact that mutating His-493 does not affect HSL activities strongly suggests that the 17.6 kDa fragment proposed by others to be the catalytic core [15] cannot have catalytic activity per se. The PNPB hydrolyzing activity described for this fragment [15] may in fact be due to the interaction of several proteolytic fragments, held together by inter-atomic forces even after the cleavage of the peptide chain [9].

In order to verify the correct expression of the different HSL constructs, Western blot analyses were performed on the cell supernatants. Fig. 3 shows a representative Western blot, with one lane for each construct. Wild-type HSL and all HSL mutants appear as an 84 kDa band, as expected, and this band is absent in supernatants of cells transfected with pSVL alone. Interestingly, the HSL bands corresponding to the Asp-703 mutants are weaker than those obtained for the rest of the mutants and the wild-type (≈30% of wild-type HSL; Fig. 3). This observation was consistent in all experiments performed, and probably reflects a reduced stability of the mutant protein

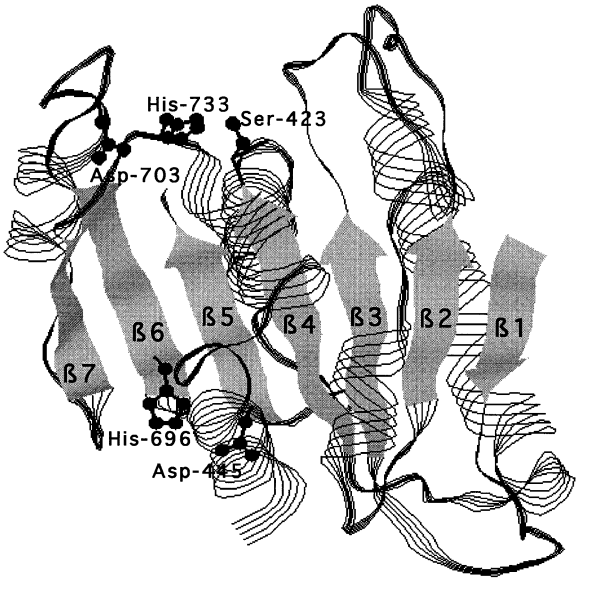


Fig. 1. Schematic representation of the structural model of HSL C-terminal catalytic domain. The expected catalytic triad and other residues that were mutated in this work are shown in ball-and-stick representation. The figure was made with RasMol [25].

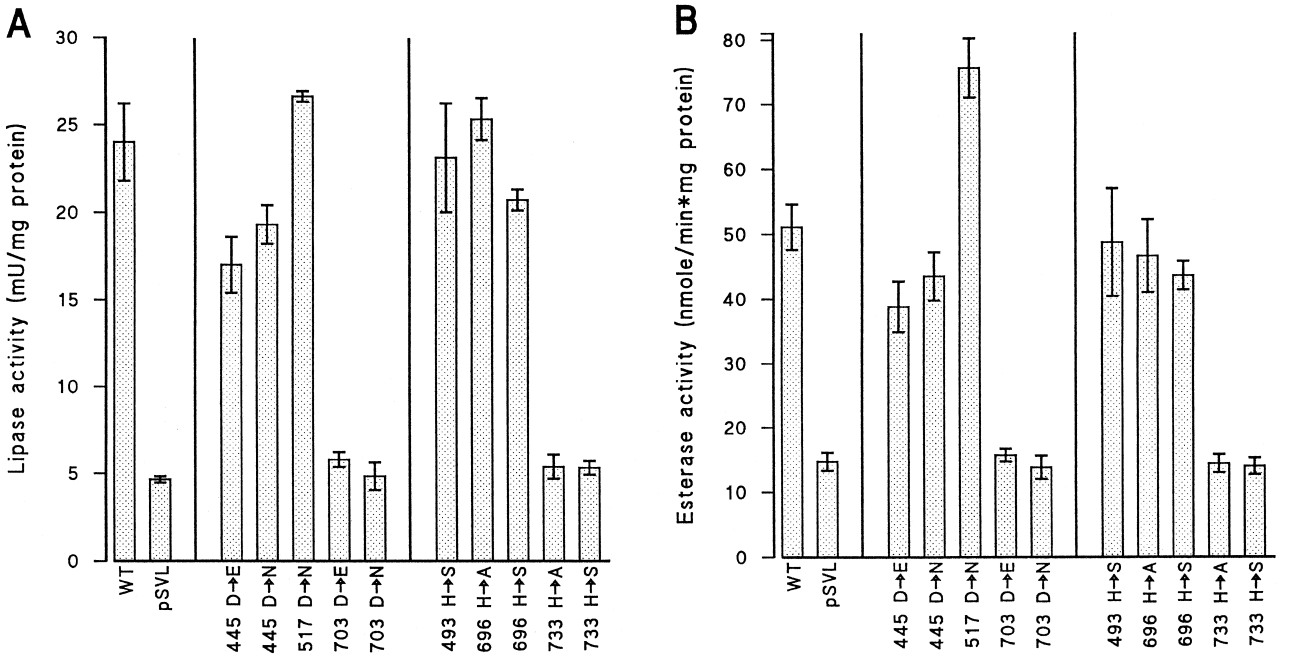


Fig. 2. Lipase and esterase activities of COS cells transfected with different HSL mutants. pSVL plasmids containing rat HSL cDNAs encoding mutations of the indicated residues were used to transfect COS cells. Three days after transfection, the cells were analyzed for lipase activity (A) and esterase activity (B). The bars represent the mean ± SD from three plates, and each determination was in triplicate. None of the mutants of residues Asp-703 and His-733 showed any significant lipase or esterase activity compared with the control cells transfected with pSVL alone ($P > 0.05$, Student's $t$-test), with the possible exception of Asp-703-Glu ($P = 0.013$). Mutants are denoted with the residue number, followed by one letter code amino acids indicating the wild-type residue and the mutation made.

in the COS cells. It is not an unusual phenomenon that the stability of lipases and esterases is affected by mutating the active site carboxylic acid. It has, for example, been observed for pancreatic lipase [21], *G. candidum* lipase [23] and acetylcholine esterase [24]. Due to their polar character, aspartic (and glutamic) acid residues are usually involved in the formation of ion and/or hydrogen bonding that contribute to the stability of the folding. Thus, the carboxylic acid of the triad participates often in bonding to several residues near the active site, including the histidine (and in some cases even water molecules), playing a role not only in catalysis, but also in stabilizing the enzyme (see [12]). In our structural model for the catalytic domain of HSL it can be foreseen that Asp-703 may form hydrogen bonds to several close residues, similar to what is observed in the crystal structure of *G. candidum* lipase, *C. rugosa* lipase and *T. californica* acetylcholine esterase.

In summary, we have herein probed different candidate residues for the catalytic triad of HSL by site-directed mutagenesis. The results clearly show that Asp-703 and His-733 are essential for the lipase and esterase activities of HSL, whereas Asp-455 and His-493 are not. These results provide experimental support that Asp-703 and His-733, together with the previously identified Ser-423 [7], constitute the catalytic triad of HSL, as proposed in our structural model for the catalytic domain of HSL.

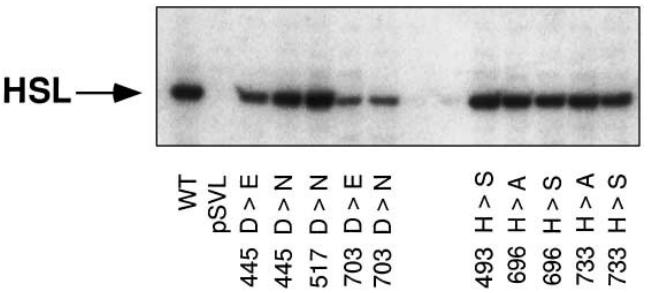


Fig. 3. Western blot analysis of COS cells transfected with different HSL mutants. Aliquots of the supernatants from transfected COS cells corresponding to 30 μg of total protein were analyzed by SDS-PAGE and Western blot, as indicated in Section 2. Mutants are denoted as in Fig. 2.

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