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Identification of the active site serine of hormone-sensitive lipase by site-directed mutagenesis

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Abstract

The consensus pentapeptide GXSXG is found in virtually all lipases/esterases and generally contains the active site serine. The primary sequence of hormone-sensitive lipase contains a single copy of this pentapeptide, surrounding Ser-423. We have analyzed the catalytic role of Ser-423 by site-directed mutagenesis and expression of the mutant hormone-sensitive lipase in COS cells. Substitution of Ser-423 by several different amino acids resulted in the complete abolition of both lipase and esterase activity, whereas mutation of other conserved serine residues had no effect on the catalytic activity. These results strongly suggest that Ser-423 is the active site serine of hormone-sensitive lipase.

Key words: Lipase; Active site; Site-directed mutagenesis

1. Introduction

Hormone-sensitive lipase (HSL; EC 3.1.1.3.) catalyzes the rate-limiting step in the hydrolysis of stored triacylglycerols and is therefore a key enzyme in lipid metabolism and overall energy homeostasis [1,2]. Its activity is under acute neural and hormonal control, exerted via phosphorylation of a single serine residue (Ser-563 in rat HSL) by cAMP-dependent protein kinase [3,4]. Lipolytic hormones, such as catecholamines, cause a phosphorylation and concomitant activation of HSL, whereas antilipolytic hormones, of which insulin is the most important, cause a net dephosphorylation and deactivation of HSL.

HSL in the rat is a protein of 768 amino acids, which shows no significant overall homology with any of the other sequenced mammalian lipases [3,5]. In particular, it shares no obvious sequence homology with the members of the lipase gene family, i.e. pancreatic lipase, lipoprotein lipase and hepatic lipase. However, HSL has several biochemical and functional features in common with these lipases. For instance, both HSL and lipoprotein lipase hydrolyze long-chain triacylglycerols, have similar positional specificity, are inhibited by serine-directed reagents and most likely function as dimers [1,2,6]. On the other hand, HSL has some unique features. The most noticeable is its dramatic activation upon phosphorylation by cAMP-dependent protein kinase, and its ability to hydrolyze cholesteryl esters [1,2]. Moreover, it has been shown that HSL shows a relatively higher catalytic activity at low temperatures than both lipoprotein lipase [6] and carboxyl ester lipase [7]. This property was discovered after an unexpected homology between HSL and lipase 2 of *Moraxella* TA 144, an antarctic, psychrotrophic bacterium, had been found [5,8]. It may reflect a cold-adaptability property of HSL [5] and could be of vital importance in hibernators and poikilotherms. The sequence relationship found between HSL and the *Moraxella* lipase was recently extended to include several other bacterial proteins from distantly related eubacteria [9,10], and it was suggested that HSL is closely related to a group of bacterial proteins, and only very distantly related to lipoprotein lipase, hepatic lipase and pancreatic lipase [10].

In common with virtually all other hitherto sequenced mammalian and microbial lipases and esterases [11,12], HSL contains a GXSXG motif, in this case surrounding Ser-423 [3,5]. Site-directed mutagenesis has been used to show that the pentapeptide serine is analogous to the active site serine in several lipases, including hepatic lipase, lipoprotein lipase and pancreatic lipase [13–17]. Structural evidence for the catalytic site serine of these related lipases was obtained upon determination of the crystal structure of pancreatic lipase, which revealed that the serine of this motif is part of a catalytic triad also

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Abbreviations: cDNA, complementary DNA; DFP, diisopropyl fluorophosphate; HSL, hormone-sensitive lipase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, polyacrylamide gel elecrophoresis in sodium dodecyl sulphate.

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including an aspartic acid and a histidine [18]. However, the GXSXG motif has a wide occurrence and is found in approximately every tenth protein in the existing databases, including many proteins with no hydrolytic function [12]. This fact, together with the observation that HSL seems to be closely related to some not very well-characterized bacterial proteins rather than to the mammalian lipases, where the GXSXG is known to contain the active site serine, prompted us to investigate the functional role of Ser-423.

We have used site-directed mutagenesis to produce mutants with conservative substitutions for Ser-423. In addition, other serine residues, randomly picked, but conserved between rat and human HSL, were mutated to serve as controls. Activities of the resulting mutants are consistent with the conclusion that Ser-423 is the catalytic serine in HSL.

2. Materials and methods

2.1. Construction of mutants

Mutations were introduced into rat HSL cDNA using the PCR overlap extension method [19] and Vent DNA polymerase with proofreading activity (New England Biolabs.) to amplify sequences from full-length rat HSL cDNA in the pSVL vector [20]. Briefly, each mutant construct was composed of two PCR fragments, joined at the site of the mutation, and together encompassing the complete rat HSL coding sequence. Primers for the C-terminal fragment consisted of a 27-mer containing the desired mutation as its central codon and a 27-mer containing the reverse complement of the cDNA 8 nucleotides downstream of the stop codon. Similarly, the N-terminal fragment was flanked by primers beginning at the ATG start codon and ending with reverse complement of the mutant oligo described above. The two PCR fragments were purified by Centricon 100 (Amicon), separated from the template by agarose gel-electrophoresis and joined in a final PCR reaction using overlap extension. The final product was purified by Centricon 100, digested with *Xho*I and *Sac*I (sites for these enzymes were included in the end primers), isolated by agarose gel-electrophoresis and then ligated into pSVL. A cDNA without mutations was also constructed using Vent DNA polymerase and the same cDNA template and end primers as above. This was ligated into pSVL and used as control DNA in the transfection experiments (referred to as wild-type DNA).

2.2. DNA sequencing

The identity of each mutant and the absence of PCR mistakes in the inactive HSL Ser-423 mutants was confirmed by dideoxynucleotide sequencing of double-stranded DNA using the *Taq* Dyedoxy terminator cycle sequencing kit (Applied Biosystems) and a model 373A DNA sequencer (Applied Biosystems). Besides the introduced mutations, some differences from the reported cDNA sequence [3] were observed in all mutants, and also in the wild-type DNA (see above). First of all, as detected previously (Z. Li, M. Sumida, A. Birchbauer, M.C. Schotz and K. Reue, personal communication), at position 2850 [21] an extra C was found, which changes and extends the C-terminal region of the deduced amino acid sequence from GRPRSCACSASG to RQAAELCVQRIRLILTPPAAPLT. Secondly, in the sequence coding for the region immediately upstream of the phosphorylation sites, which is predicted to be a connecting loop in the rat protein but is absent in the human protein [5], a reported G at position 2239 [21] could not be confirmed, and at position 2281 [21] an extra C was found. This changes the deduced amino acid sequence in the region from amino acids 542–556 from VACNRDTAPHGFWAL to LPATETPRPTDSGRL. The observed differences from the reported cDNA sequence [3,21] have been confirmed by re-sequencing the original cDNA clones from the λgt11 library [3,21] subcloned into pUC19.

2.3. Transfection and expression of HSL cDNA

Transfection of COS cells by lipofectin (BRL) was performed as previously described using 1 μg of DNA per 60 mm dish [20]. Cells were harvested after 72 h by centrifugation in PBS, followed by homogenization in 0.25 M sucrose, 1 mM EDTA, 1 mM dithioerythritol, 20 μg/ml leupeptin, 2 μg/ml antipain and 1 μg/ml pepstatin at 4°C. Total protein in the cell homogenates was measured according to Bradford [22] using bovine serum albumin as the standard.

2.4. Western blot analysis

The HSL protein concentration in the cell homogenates was estimated by subjecting aliquots of the different homogenates to SDS-PAGE, followed by Western blot analysis using a polyclonal HSL antibody, anti-rabbit IgG, conjugated to alkaline phosphatase, and a chromogenic substrate (Protoblot II AP Systems; Promega, Madison, WI) [23,24].

2.5. HSL assay

The lipase activity of homogenized cells was measured using a phospholipid-stabilized emulsion of 1 (3)-mono [^{3}H]oleoyl-2-oleylglycerol, a diacylglycerol ether analogue [25]. HSL activity is expressed in mU where 1 mU = 1 nmol fatty acid released per min at 37°C.

2.6. Esterase assay

Activity toward water-soluble substrate was measured using *p*-nitrophenylbutyrate [26]. Briefly, aliquots of the homogenized cells were incubated in 1 ml of 0.1 M NaH_2PO_4, 0.9% NaCl, 1 mM dithioerythritol and 0.5 mM *p*-nitrophenylbutyrate for 10 min at 37°C. Enzyme reactions were terminated by the addition of 3.25 ml methanol/chloroform/heptane (10:9:7) to form a two-phase partition system. Absorbance of the *p*-nitrophenol-containing supernatant was measured at 400 nm.

3. Results and discussion

The serine of the GXSXG motif in rat HSL, i.e. Ser-423, was mutated to either Gly, Ala, Thr or Cys (Table 1). These substitutions for serine have been shown to minimize disturbance in protein structure [27]. Four other serine residues, randomly picked but conserved between the rat and human sequence [3,5], were also mutated (Table 1). The different HSL cDNAs were subcloned in pSVL and expressed in COS cells. Homogenates of harvested cells were analyzed for expression of HSL protein by Western blot analysis with an anti-rat HSL antibody. All the different mutants were shown to be expressed to approximately the same level as wild-type HSL (Fig. 1).

The lipase activity of the different mutants were measured in the cell homogenates using a diacylglycerol ana-

Table 1
Site-specific mutations in HSL

Plasmid	Amino acid	Resultant amino acid
pSVL/423-SG	Ser-423	Gly
pSVL/423-SA	Ser-423	Ala
pSVL/423-SC	Ser-423	Cys
pSVL/423-ST	Ser-423	Thr
pSVL/185-SA	Ser-185	Ala
pSVL/320-SA	Ser-320	Ala
pSVL/412-SA	Ser-412	Ala
pSVL/533-SA	Ser-533	Ala

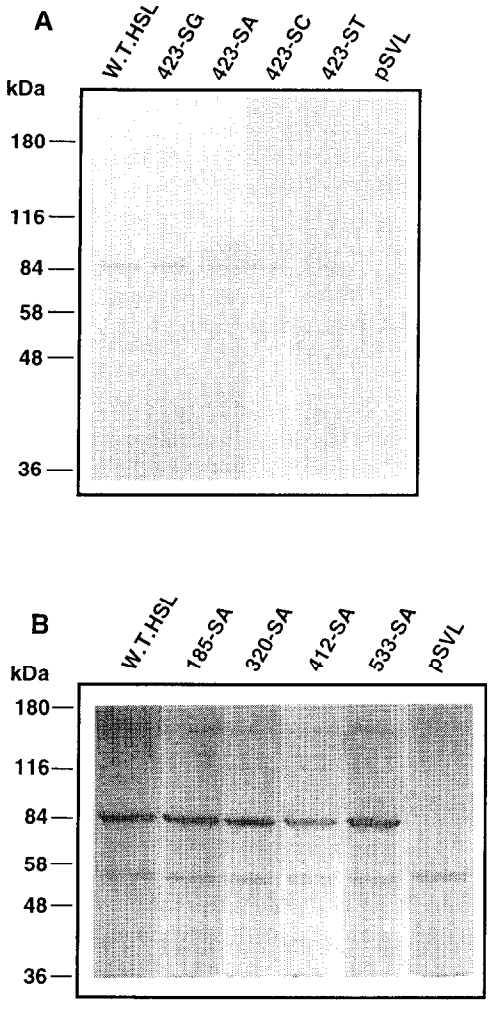

Fig. 1. Expression of HSL mutants in COS cells. (A) pSVL plasmids containing HSL cDNAs encoding mutations of Ser-423 to Gly, Ala, Cys or Thr, and (B) pSVL plasmids containing HSL cDNAs encoding mutations of Ser-185 to Ala, Ser-320 to Ala, Ser-412 to Ala and Ser-533 to Ala, Gly, Cys or Thr, were used to transfect COS cells. Aliquots of the cell homogenates, corresponding to equal amounts of total protein, were analyzed by SDS-PAGE and Western blot analysis, as described in section 2. The different mutations are denoted as follows: SG, glycine; SA, alanine; SC, cysteine; ST, threonine. W.T.HSL, wild-type HSL; pSVL, the pSVL vector alone without HSL cDNA.

log as substrate. None of the Ser-423 mutants had any significant lipase activity above that of cells transfected with the pSVL vector alone (Fig. 2A) whereas the activities of other serine mutants were not significantly different from the wild-type HSL (Fig. 3).

To distinguish between an effect due to a mutation in the catalytic site from a mutation in the interfacial binding site, esterase activity measurements were performed against *p*-nitrophenylbutyrate. This substrate, in contrast to the lipid emulsion used above, does not form interfaces. Also using this substrate, the Ser-423 mutants were shown not to have any significant activity compared with that of cells transfected with the pSVL vector alone (Fig. 2B).

These results strongly suggest that Ser-423 is the active site serine of HSL. The fact that the Ser-423 mutants were devoid of both lipase and esterase activity rules out the possibility that Ser-423 is part of an interfacial binding site rather than the catalytic site. This is also in agreement with what has been found for the serine of the GXSXG motif of the other mammalian lipases [13–18].

Much has been learnt regarding the catalytic site of lipases from the determination of the three-dimensional structure of pancreatic lipase [18] and several microbial lipases, such as the *Rhizomucor miehi* lipase [28] and the *Geotrichum candidum* lipase [29]. The three-dimensional structure of these lipases have shown that the serine of the GXSXG motif is part of a catalytic triad, including

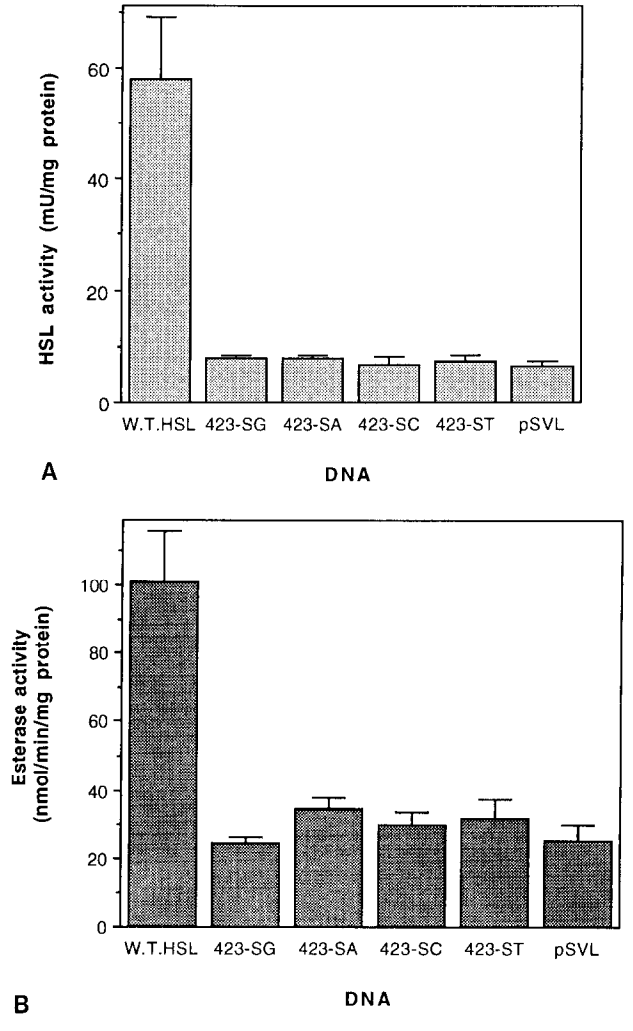

Fig. 2. Transfection of COS cells with HSL (Ser-423) mutants. 1 μg each of pSVL plasmids containing HSL cDNAs encoding a mutation of Ser-423 to Gly, Ala, Cys and Thr, respectively, were used to transfect COS cells. Cell homogenates were analyzed for HSL lipase activity, esterase activity and total protein. Shown is the HSL activity in mU/mg protein (A) and the esterase activity in μmol *p*-nitrophenol released per min and μg protein (B). The bars represent the mean ± S.D. from three 60 mm plates for each construct. None of the mutants were significantly different from pSVL alone ($P > 0.01$) by Student's *t*-test. The different mutations are denoted as in Fig. 1.

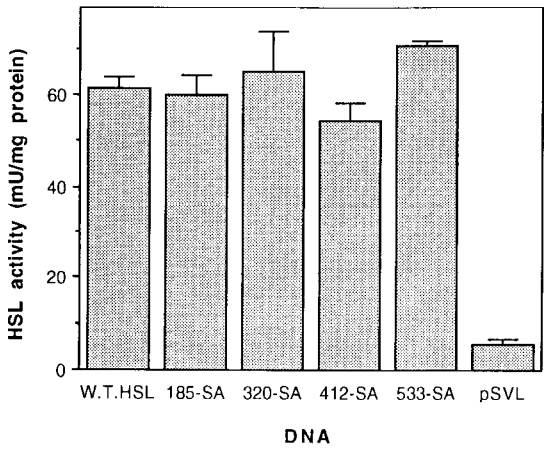

Fig. 3. Transfection of COS cells with HSL cDNAs encoding mutations in some conserved serine residues. pSVL plasmids (1 μg/dish) containing HSL cDNAs encoding mutations of Ser-185 to Ala, Ser-320 to Ala, Ser-412 to Ala and Ser-533 to Ala, Gly, Cys or Thr, respectively, were used to transfect COS cells. Cell homogenates were analyzed for HSL lipase activity and total protein. Shown is the HSL activity in mU/mg protein. The bars represent the mean ± S.D. from three 60 mm plates for each construct. None of the mutants were significantly different ($P > 0.05$) from wild-type HSL by Student's t-test. The different mutations are denoted as in Fig. 1B.

also an aspartic acid, or in the case of *Geotrichum candidum* lipase a glutamic acid, and a histidine. In all these three lipases the catalytic triad, situated in a pocket, is covered by one or two surface loops or lids. Co-crystallization experiments with inhibitors and cofactors have shown for both *Rhizomucor miehi* lipase and human pancreatic lipase that during interfacial activation the lid is displaced, uncovering the catalytic site, and inducing other conformational rearrangements that create a hydrophobic binding site for the substrate as well as the oxyanion hole [30,31]. Since the catalytic triad of pancreatic lipase is known, it has been possible, by sequence analogy, to assign the active site aspartic acid and histidine of lipoprotein lipase [16]. However, in contrast to the active site serine, there is no strong consensus sequence environment for the active site aspartic acid or histidine, making it difficult to identify these residues in lipases lacking sequence identity to lipases with known three-dimensional structures.

Since the GXSXG motif is found in virtually all microbial and mammalian lipases/esterases with known primary structures, it is reasonable to assume that the GXSXG motif of these proteins is derived from a common ancestral gene. In this context, it can be noted that different functional regions of HSL are encoded by different exons. The GXSXG motif is encoded by exon 6, the regulatory phosphorylation site by exon 8 and a putative lipid binding site by exon 9, thus suggesting that HSL is a mosaic protein [5]. The GXSXG motif is also found around the active site serine of serine proteases. However, it has been shown for serine proteases of the chymotrypsin and subtilisin family that the structure around the GXSXG motif is very different from that of the lipases, and therefore they may not be related except by convergent evolution [12]. The glycines of the motif appear to be invariant in the lipases due to packing considerations and not due to restraints imposed by secondary structure, which is the case in the chymotrypsin and subtilisin family. Furthermore, the serine of the motif adopts the very unusual ε-type conformation in the lipases, which is not the case in the proteases [12], and the internal order between the residues of the catalytic triad is different between the lipases and serine proteases. A recently described family of serine proteases, the prolyl oligopeptidase family, however, has been suggested to be related to the lipases, based on the finding that they seem to have the catalytic residues in the same order as the lipases, and thus the reverse order of what is found in the chymotrypsin and subtilisin family [32].

In summary, we have used mutagenesis experiments to show that Ser-423 of rat HSL is the active site serine. This is the first demonstration in HSL of a relationship between catalytic function and structure. Future experiments solving the three-dimensional structure of HSL and more mutagenesis experiments will allow a more detailed description of the active site and the regions important for interaction with the lipid substrate.

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