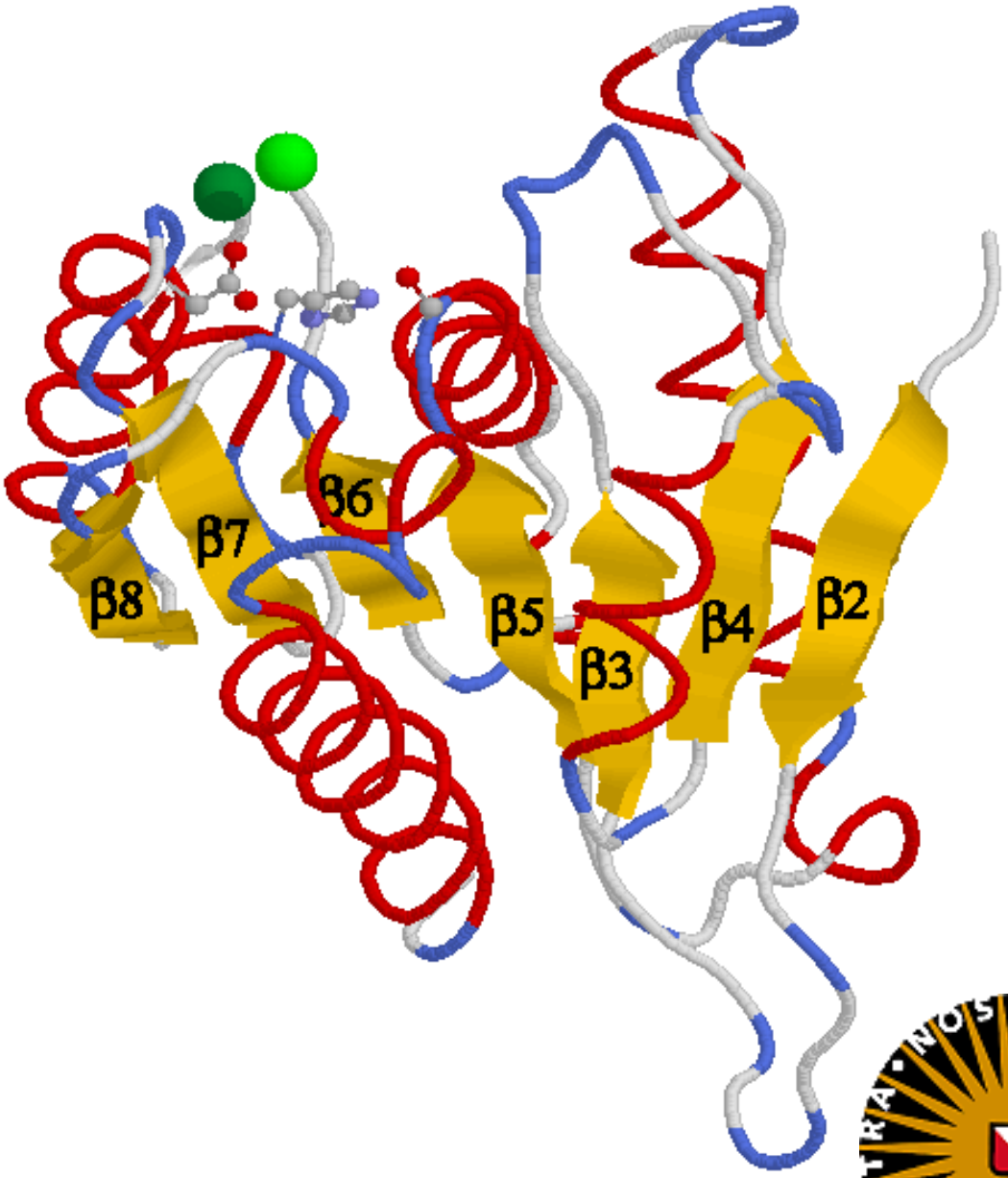
Hormone sensitive lipase: structure, function and regulation



Jeroen de Meijer
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Front page illustration:
Hormone sensitive lipase (kindly provided by J.A. Contreras and C. Holm)

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J. de Meijer 1998

A thesis writen at the Biochemical Physiology Research Group, Department of Experimental Zoology, University of Utrecht, under supervision of dr. W. J. A. van Marrewijk

Abstract

Hormone sensitive lipase (HSL) is the key enzyme in the regulation of lipid stores. It is the rate limiting enzyme in the degradation of triacylglycerol (TAG) to diacylglycerol (DAG) and free fatty acids (FFA). In addition, it has hydrolyzing activity against cholesterol esters. In this sight it is not remarkable that HSL is not only found in adipose tissue, but also in tissues that store cholesterol esters. In these tissues HSL plays a key role in the cholesterol metabolism.

HSL is regulated by reversible phosphorylation on four residues. Phosphorylation alone, however, is not enough to activate HSL. It probably also involves conformational changes and a translocation from the cytoplasm to the lipid droplet.

HSL is a product of the HSL gene, located in human on chromosome 19, which contains 9 exons. Every exon (or exons) gives rise to a distinct domain of HSL suggesting that the HSL gene is a mosaic gene. The promoter of the HSL gene has only recently been characterized and has no TATA or CCAAT box. It does contain the consensus sequences common in TATA-less promoters like a GC-rich region, an AT-rich region and an initiator region. The regulation of the gene remains obscure at this moment.

The HSL protein has an α/β hydrolase fold conformation. It contains a catalytic triad commonly found in lipases. The regulatory phosphorylation sites are located on a loop protruding the protein. The carboxyl terminal and/or the amino terminal can have a role in lipid binding.

The knowledge about HSL is still premature but is growing rapidly. Not remarkably, as knowledge about lipid metabolism is of crucial importance in several health issues today.

Contents

1 Introduction

Every organism continuously uses energy; not only for movement or to perform labor, but also for maintenance, regulation and almost any other process. Organisms derive their energy from the breakdown of nutrients they take up. The type of nutrients can roughly be divided in three groups: proteins, carbohydrates and lipids. During breakdown of these nutrients energy is produced in the form of ATP, which is the universal energy carrier in organisms. Most nutrients, however, are not directly used for energy, but are stored in tissues in an appropriate form for later use.

Proteins are normally not used for energy but broken down into amino acids which can be used to construct new proteins. Only in case of a severe lack of energy sources proteins are used for energy. In that case the proteins are broken down into alanine and glutamine and processed into glucose by the liver and the kidney respectively. This is important in the brain as this tissue can not utilize lipids as an energy source.

Carbohydrates are, however, a major energy source. Used carbohydrates are mostly sugars like glucose or trehalose. These sugars

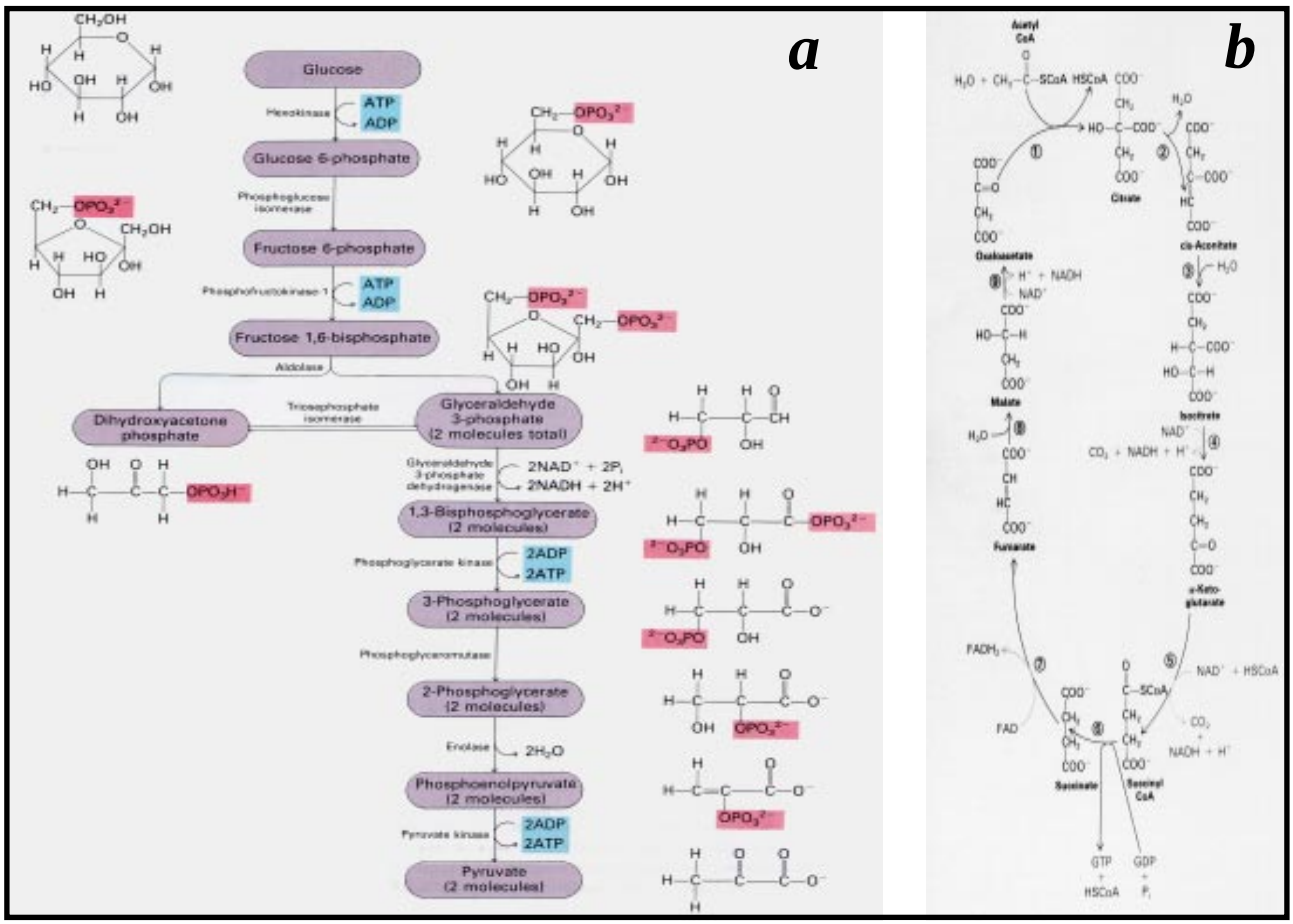
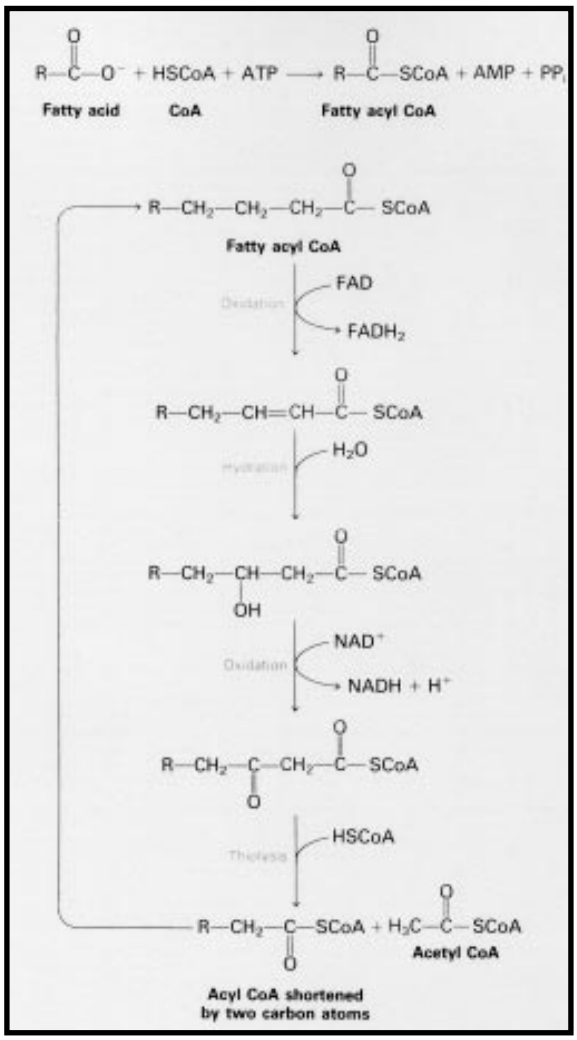


***Figure 1**;*
a) The glycolytic pathway by which glucose is degraded to two molecules of pyruvate. Along the way two ATP molecules and two molecules NADH+H$^+$ are formed. Pyruvate is subsequently converted into acetyl-CoA by the pyruvate dehydrogenase complex (not in figure). In this process one molecule of NADH+H$^+$ is formed and one molecule CO_2.
b) The citric acid cycle. A two carbon acetyl residue from acetyl-CoA condenses with oxaloacetate (1) to form citrate. In a cascade of reactions (2-9) citrate is converted in oxaloacetate which can subsequently condense again with a two carbon acetyl residue from acetyl-CoA. Every cycle produces three molecules NADH+H$^+$, one molecule $FADH_2$, one molecule GTP, and 2 molecules CO_2.
NADH+H$^+$, $FADH_2$ and GTP are used to produce ATP, the overall energy carrier in the cell. (Adapted from Lodish et al. 1995)

***Figure 2**;*
Oxidation of fatty acids. Four reactions convert a fatty acyl-CoA to acetyl-CoA and a fatty acyl-CoA shortened by two carbon atoms. Thus, to breakdown a 16-carbon fatty acid (palmitate) seven cycles are neccesary. The produced acetyl-CoA will enter the Krebs cycle (Figure 1b). (Adapted from Lodish et al. 1995)



are, in mammals, stored as glycogen in the liver. When needed, the glycogen is broken down into sugars and released to the circulation system. A key regulator in this breakdown is the enzyme glycogen phosphorylase. Carbohydrates provide a quick energy source and are degraded in the well known glycolytic pathway and the subsequent citric acid cycle (***Figure 1***). The pool of available carbohydrates is, however, quickly exhausted, making carbohydrates insufficient as an energy source for long endurances. And this is where lipids come into play.

Lipids are mostly stored as triacylglycerol (TAG) in the adipose tissues and are the most important energy store in mammals. When needed TAG is broken down to diacylglycerol (DAG) or free fatty acids (FFA) and glycerol. The FFA are broken down in the β-oxidation cycle (***Figure 2***). Key regulators in the breakdown of TAG are monoacylglycerol lipase and hormone sensitive lipase (HSL) (Yeaman *et al.* 1989). HSL catalyzes the rate-limiting step in this hydrolysis of TAG and is therefore the focus of this thesis.

2 Physiological role of hormone sensitive lipase

The use of energy stores is, to meet the continuously changing demand for energy, tightly regulated depending mainly on neural and hormonal signals.

During activity energy sources are modulated to ensure the availability of the appropriate form of energy. For instance, during long endurances, the source switches from the "fast" energy (carbohydrates) to the "slow" energy (lipids). Upon starvation this switch is also seen. Only the brain keeps using carbohydrates as it can not metabolize lipids. When starvation proceeds, proteins are used for energy and also ketone bodies are formed which the brain can use for energy. It will be clear that regulation is of crucial importance. Deregulation can have a serious impact on the health of the organism, for instance obesity or diabetes.

As stated before, HSL is a key enzyme in the regulation of the most important energy source: lipids. HSL is, as the name implies, hormonally regulated. In response to various lipolytic hormones HSL is phosphorylated and activated. This phosphorylation is reversible, for instance in response to anti-lipolytic hormones like insulin (Yeaman 1990).

TAG has three ester bonds which can be hydrolyzed, resulting in FFAs (***Figure 3***). Although HSL is capable of fully hydrolyzing TAG to glycerol and FFA, HSL has a marked specificity for the 1(3)-ester bond (Fredrikson *et al.* 1983). There is evidence that the

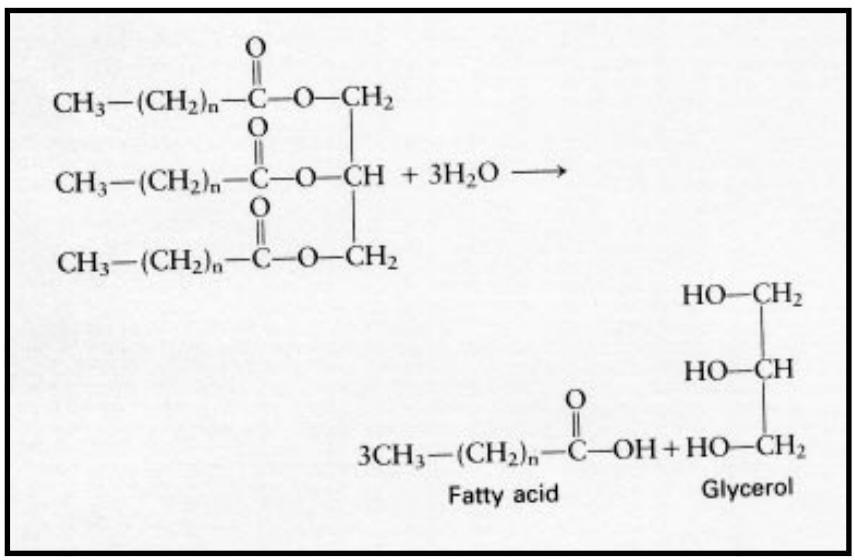


Figure 3;
Triacylglycerol consists of a glycerol backbone and three lipid residues. The bond can be hydrolyzed to produce free fatty acids and glycerol. (Adapted from Lodish et al. 1995)

2-ester bond is hydrolyzed by monoacylglycerol lipase and that this enzyme is required for an efficient complete hydrolysis of TAG (Fredrikson *et al.* 1986).

HSL, however, not only possesses activity against TAG, DAG, and monoacylglycerol (MAG) but also against the long chain esters of cholesterol. Remarkably, the activity of HSL against cholesterol is approximately equal to its activity against TAG (Fredrikson *et al.* 1981). This suggests an additional role for HSL in cholesterol metabolism, beside the hydrolysis of lipids.

As one would aspect from its dual role, HSL is not only present in adipose tissues but is also found in tissues in which cholesterol esters are stored. For instance adrenal cortex, ovaries, heart (Small *et al.* 1989b), muscle and macrophages. The first evidence for this was found when 84 kDa neutral cholesterol ester hydrolase was found in bovine adrenal cortex tissue (Cook *et al.* 1981). The activity of this hydrolase, like HSL, increased in the presence of cyclic AMP-dependent protein kinase (Beckett *et al.* 1977). Later it was confirmed that this hydrolase was indeed HSL (Cook *et al.* 1982). In addition, HSL was found in the corpus luteum which is also a tissue that produces steroid hormones (Cook *et al.* 1983). The theory is postulated that the role of HSL in these steroid producing tissues is the supply of free cholesterol which is the precursor for steroidogenesis. The role of HSL in macrophages will be discussed later.

Summarizing, roughly three roles can be distinguished:

- lipid metabolism
- cholesterol metabolism
- steroidogenesis

3 Regulation of hormone sensitive lipase

In contrast to the regulation of the energy stored in carbohydrates, much less is known about the regulation of the lipid energy stores. HSL plays a key role in this lipid metabolism. This hydrolyzing enzyme is regulated by reversible phosphorylation. This reversible phosphorylation is under tight hormonal control, and as a result so is HSL. When rat adipocytes were treated with lipolytic agents (like noradrenaline) HSL phosphorylation was increased (Belfrage *et al.* 1980). When these adipocytes were treated with the known anti-lipolytic agent, insulin, HSL showed a decrease in phosphorylation (Nilsson *et al.* 1980). Phosphorylation of HSL will be discussed in more detail in chapter 5.

The regulation of HSL is not solely on the level of protein phosphorylation as phosphorylation of HSL does not seem to be sufficient to obtain a high level of HSL activity. There is a dramatic difference between the activity of phosphorylated HSL *in vitro* and *in vivo*: the activity *in vitro* being substantially lower (Nilsson *et al.* 1980, Fredrikson *et al.* 1981, Cook *et al.* 1982, Strålfors *et al.* 1983).

There is evidence that a translocation, possibly induced by phosphorylation, is necessary to yield a high HSL activity. This will be discussed in more detail in chapter 6. In addition, accessory proteins, like perilipin discussed in chapter 7, seem to be involved in the HSL regulation. Beside this, also the composition of the substrate droplet seems to effect the activity of HSL. Okuda *et al.* (1994) have shown that the activity of HSL is greatly influenced by the phospholipid content of the lipid droplet surface. They also showed that hormones can act on endogenous fat as substrate (Okuda *et al.* 1983, 1986). If hormones change the interfacial properties of the lipid droplet it could effect the liplytic activity of the fat cell. The presence of phosphatidylcholine on the droplet surface greatly reduced the responsiveness of the lipid droplets to HSL. Phospholipase C seems to restore the responsiveness.

HSL is possibly also subject to regulation at the gene level. In the mouse HSL gene regulatory elements have been found in the 5' flanking region that controls expression in specific tissues (Li *et al.*

1994). In addition a sterol regulatory element has been found in HSL which regulates the transcription of several genes encoding proteins involved in cholesterol metabolism (Goldstein *et al.* 1990). Very recently, Laurell *et al.* (1997) showed that HSL is subject to species-specific alternative splicing, generating a short form of HSL mRNA. This mRNA is a result of skipping exon 6 which contains a part of the catalytic triad necessary for HSL activity. They postulate that this alternative splicing of HSL mRNA could have a role in the fine regulation of HSL as this splicing decreases the amount of mRNA that can be translated into functional HSL and thereby reduces the amount of functional HSL.

Before investigating the regulation of HSL in more detail, we will first discuss the structure of the HSL gene and protein.

4 Structure of the hormone sensitive lipase gene and protein

4.1 Homology

Most enzymes can be categorized in families. At first, however, HSL did not seem to be a member of the known family of mammalian lipases which includes lipases as lipoprotein lipase , hepatic lipase and pancreatic lipase (Cordle *et al.* 1986, Kirchgessner et al. 1986). Virtually no homology was found with any mammalian protein (Langin *et al.* 1993, Li *et al.* 1994). Only one lipase showing amino acid sequence homology with HSL was found so far: the cold-adapted lipase 2 of *Moraxella* TA144 (Langin *et al.* 1993a). This suggests that HSL could also have cold-adaptability properties. This idea is supported by the observation that HSL shows a relatively high activity at low temperatures (Langin *et al.* 1993a). There are, however, also similarities found, in the same regions as with *Moraxella* TA144, between HSL and a prokaryotic enzyme which was found in hot springs *Bacillus acidocaldarius* (Langin *et al.* 1993b). Recently, however, Contreras *et al.* (1996) found a remarkable secundary structure homology of the HSL protein with the family of lipases and esterases. Recently it is thought that HSL is a member of an esterase subfamily of a newly described superfamily of lipases/esterases, described by Hemilä *et al.* (1994).

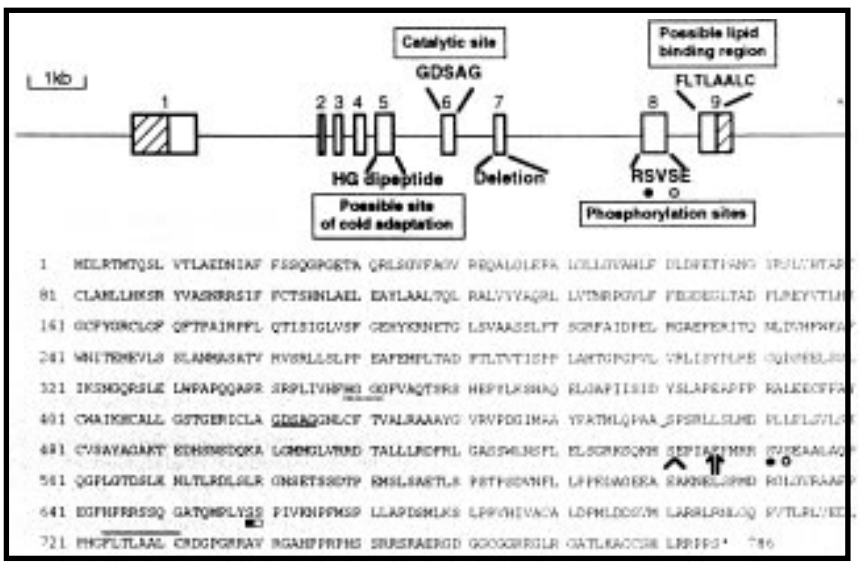


***Figure 4**;*
Organization of the human HSL gene and amino acid sequence of human HSL. The top part illustrates the exon-intron organization of the human HSL gene. Exons are represented by boxes, intron by lines. Hatched areas are noncoding regions. Different functional regions are encoded by different exons as indicated. The bottom part illustrates the aminoacid sequence of human HSL. The catalytic site serine (solid line), HG dipeptide (dotted line), the regulatory and basal phosphorylation sites (closed and open circle respectively), the newly identified phosphorylation sites (closed and open square) and the putative lipid binding region (overlined) are indicated. The deletion in human HSL compared to rat HSL is indicated with a ^, and the exon 7/8 boundary is indicated by arrows. (Adapted from Holm et al. 1994)

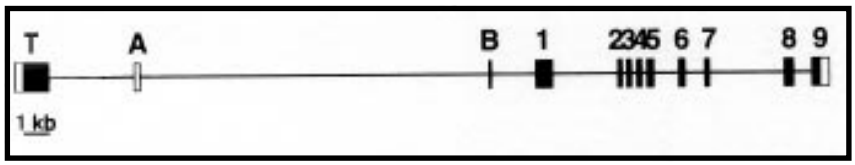


Figure 5;
Organization of the human HSL gene. Closed boxes are coding regions whereas open boxes are untranslated regions. Exon T is a testis specific exon. Exon A containing transcripts are found in significant amounts in HT29 cells and at very low levels in adipocytes (Adapted from Grober et al. 1997)

4.2 Gene structure

The rat HSL gene encodes a 786 amino-acid polypeptide (Holm *et al.* 1988b, Langin *et al.* 1993a). The polypeptide reveals no membrane spanning region, suggesting it is a free protein not attached to a membrane (Holm *et al.* 1988a). The human HSL is composed of nine exons and is located on chromosome 19 (Holm *et al.* 1988a)(***Figure 4***). It is for 83% identical to rat HSL. It is also slightly larger compared to rat HSL, and has an 12 amino acid deletion (Langin *et al.* 1993a). Exon 6 contains the catalytic site serine motif found in almost all lipases (G-X-S-X-G). This serine is thought to be part of a catalytic triad found in many lipases together with Asp_{703} and His_{733} (Contreras *et al.* 1996). Exon 8 contains the two serine phosphorylation sites. Exon 9 could contain the lipid binding region as it encodes a hydrophobic stretch (Holm *et al.* 1994).

Only recently, any attention was payed to the regulation of the HSL gene. Grober *et al.* (1997) are probably the first to elucidate the regulatory structures of the HSL gene. They showed that upstream of exon 1, two 5' untranslated regions (UTRs) are located; exon A and B, respectively, which are mutually exclusive (***Figure 5***). Exon A containing transcripts have a very low abundance in adipocytes. They also identified the transcription start site (tss) which

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-778  gggggcgtgc atatcttagc tggggagaag gcaactgggg agtacctctt
-728  ggcaagtgac aagctgaggc ccagacaagg aatctgtgga atgacacggg
-678  tgaatgacat gggtgaggaa tctctctctc tgagcctctg tttccctgtg
-628  tgaggtggga tgccagtcac agcagggtgg gctgagaggg gtaagtggca
-578  ggtccaggtt acccagtgtg gtcacaccac ccagtaggac cgacaggcag
-528  ctcaggcccc tagctgagcc tggcactgcc cttaatcggc ttttgtcctt
-478  ccaggaggga caggcagggc tggctacctc ctctctcccc ccaacagctg
-428  gggctggctg tgccagggcc agactaggag gcgggagctg agatcactgg
-378  gcctttggct ccctgagtcc tctctgggga cagagagggc agcctggctg
-328  ggtgagaggg gacggtttgt tcaggactgg ggctaggact cctgggtcct
-278  gaaggatgaa ggggctgggg gcctggactc ctgggtctga gggaggaggg
-228  gctatgggtg tggactcctg ggttctaagg cagaggagcc tgtaacagga
-178  ctaccagttg agctgagtct gggagcaggt ggggggcaga gcagggagct

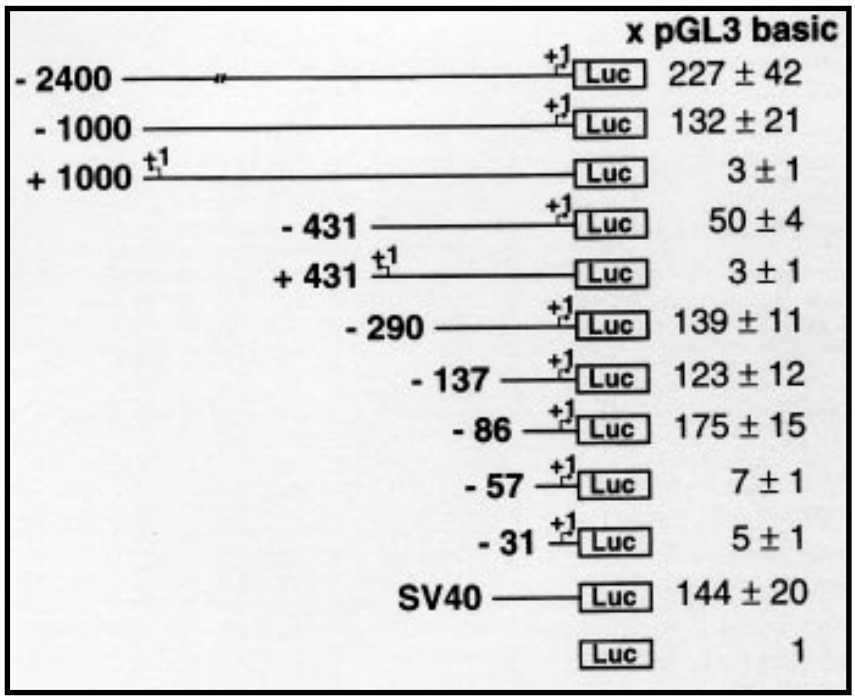
                                                      CAC
                                                      ___
-128  gagccctcta ctctgtttac agcacgtggt cctcactgat ctttctgggt

      box                                          GC box
      ___                                          ________
-78   gggaggtggc ttgtgcggct acaccctggg caggccagcc ccgccccccgg

      A+T rich
      _______                  +1
-28   gtttattgcc ccaggctgct actggcacAA GCCACAGACC AGCAGTCCCA
+23   GCCCAGGGAA GCTCGGAAGA TGCCTAGGAG GGgtgagtgt cc--intron
      (1508 bp)- acctgccccac agCCTCAAGG CTCATCCACA ACATGGACCT
```

Figure 6;
Genomic sequence of the region containing exon B. The transcription start site is indicated with +1. The putative bindings sites for transcription factors are indicated. The start codon for adipocyte human HSL is shown in italics. Capital letters indicate exonic sequences. (Adapted from Grober et al. 1997)

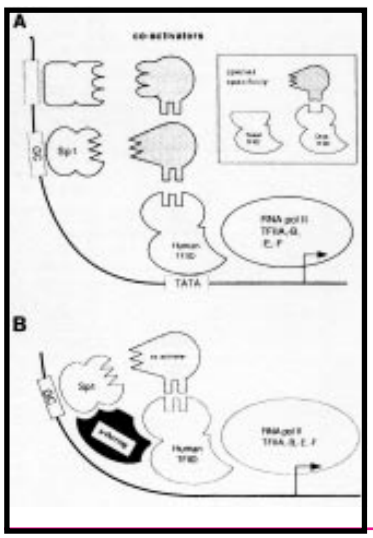
Figure 7;
Deletion analysis of human HSL promoter activity. Cells were transfected with HSL promoter-luciferase gene fusion constructs. Data are means of luciferase activities ± SEM. The data show that the 5' border of the minimum promoter is located between -86 and -57. (Adapted from Grober et al. 1997)



is located at 5' flanking region of exon B (***Figure 6***). Using truncated promoter transcripts, ranging from -2400 to -31, they determined that the 5' border of the minimal promoter is located between -86 and -57 (***Figure 7***).

Remarkably, the HSL promoter does not contain a TATA-box or a CCAAT-box, consensus sequences found in almost all promoters. This indicates that the HSL promoter is a TATA-less promoter. Several consensus sequences generally present in TATA-less promoter could indeed be found (***Figure 6***). It contains an CAC-box between -83 and -76 which can bind Sp1 and related transcription factors which possibly participate in the trans-activation of the human HSL promoter (Bucher *et al.* 1990, Boisclair *et al.* 1993). It also contains a GC-rich region which can be bound by Sp1 which probably plays a role in the stabilization of the initiation complex (***Figure 8***). It also contains a AT-rich region between -22 and -27 which could serve as a binding region for the replication complex (a replacement for the TATA-box). Also the initiator (Inr.) consensus sequence could be found in the HSL gene between -3 and +5. Although the HSL Inr. sequence differs at two positions from the Inr. consensus sequence (YYCA+1NWYY (Javahery *et al.* 1994)) this difference does not seem to disturb the promoter activity (Grober *et al.* 1997). This recent understanding of the promoter region forms the basis for further research to get a better understanding of the HSL gene regulation.

Figure 8;
Coactivator and tethering models for transcriptional activation by Sp1.
a) A model for trans-activation through coactivators. This model proposes that specific coactivators function as adapters, each serving to connect different trans-activating domains into a general transcription initiation complex.
b) Tethering model for Sp1 activation of TATA-less promoters. Sp1 correctly positions the initiation complex, with the use of a tethering factor. Also other regions can aid in this positioning (for instance the AT-rich region). (Adapted from Pugh et al. 1990)



4.3 Protein structure

Rat HSL is a polypeptide with a molecular mass of approximately 84 kDa (Belfrage *et al.* 1977, Fredrikson *et al.* 1981). Smith *et al.* (1996) have done elaborate research on the structure of HSL. Structure analysis by tryptic digestion suggests that HSL consists of separately folded domains with short, protease-sensitive, sequences in between. This analysis revealed a domain of approximately 17.6 kDa which retained its activity against the water soluble p-nitrophenyl butyrate (PNPB) but lost its activity against lipid substrates (Tsujita *et al.* 1989). This domain is located between 333 and 499 of the rat HSL sequence and contains the active site serine residue. In addition this domain contains the GXSXG motif between 421 and 425 of the rat HSL sequence which is conserved in almost all lipases. This all suggests that this domain is the catalytic domain of HSL. Additional evidence for this is provided by Holm *et al.* (1994). They have shown that substitution of serine 423 led to the complete abolition of esterase and lipase activity. Mutation of the other serine residues present in HSL had no effect. A three-dimensional model of the catalytic domain has been built by Contreras *et al.* (1996) (***Figure 9***). It consists off various α helices and β sheets folded in a so called α/β hydrolase fold, a central β sheet surrounded by a variable number of α helices. Two large differences with homologous enzymes were found. One is the connection between β7 and β8, the other the connection between β6 and β7. The latter has a major insert containing the phosphorylation sites.

Most lipases show an increase in activity in the presence of a lipid-water interface. Analysis by crystallography showed that in these lipases the catalytic triad is buried within the enzyme. On binding to the lipid interface the lid opens and exposes the active site, thereby enlarging the non-polar surface and burying the polar residues. Yeaman *et al.* (1994) have shown that this might also be true for HSL, as addition of phospholipid vesicles increased the HSL-catalyzed hydrolysis of PNPB. Trypsin treated HSL does not show an enhanced activity against PNPB in the presence of a lipid-water interface suggesting that HSL possesses a lipid binding domain that is susceptible to proteolytic digestion. The helix-loop-helix that constitutes the lid in many lipases is situated directly in

front of β2. In HSL, however, the connecting loop between β2 and the α helix in front of it (not shown in ***Figure 9***), is too short to allow the helix to cover the catalytic site. This suggests that this region is not a functional lid in HSL (Contreras *et al.* 1996).

HSL has two phosphorylation sites, termed site 1 and site 2 (respectively residue 563 and 565 of rat HSL). Tryptic digestion of HSL that is phosphorylated with ^{32}P at site 1 revealed an approximately 11.5 kDa domain which contains both phosphorylation sites (Smith *et al.* 1996). This fragment is thought to be the regulatory

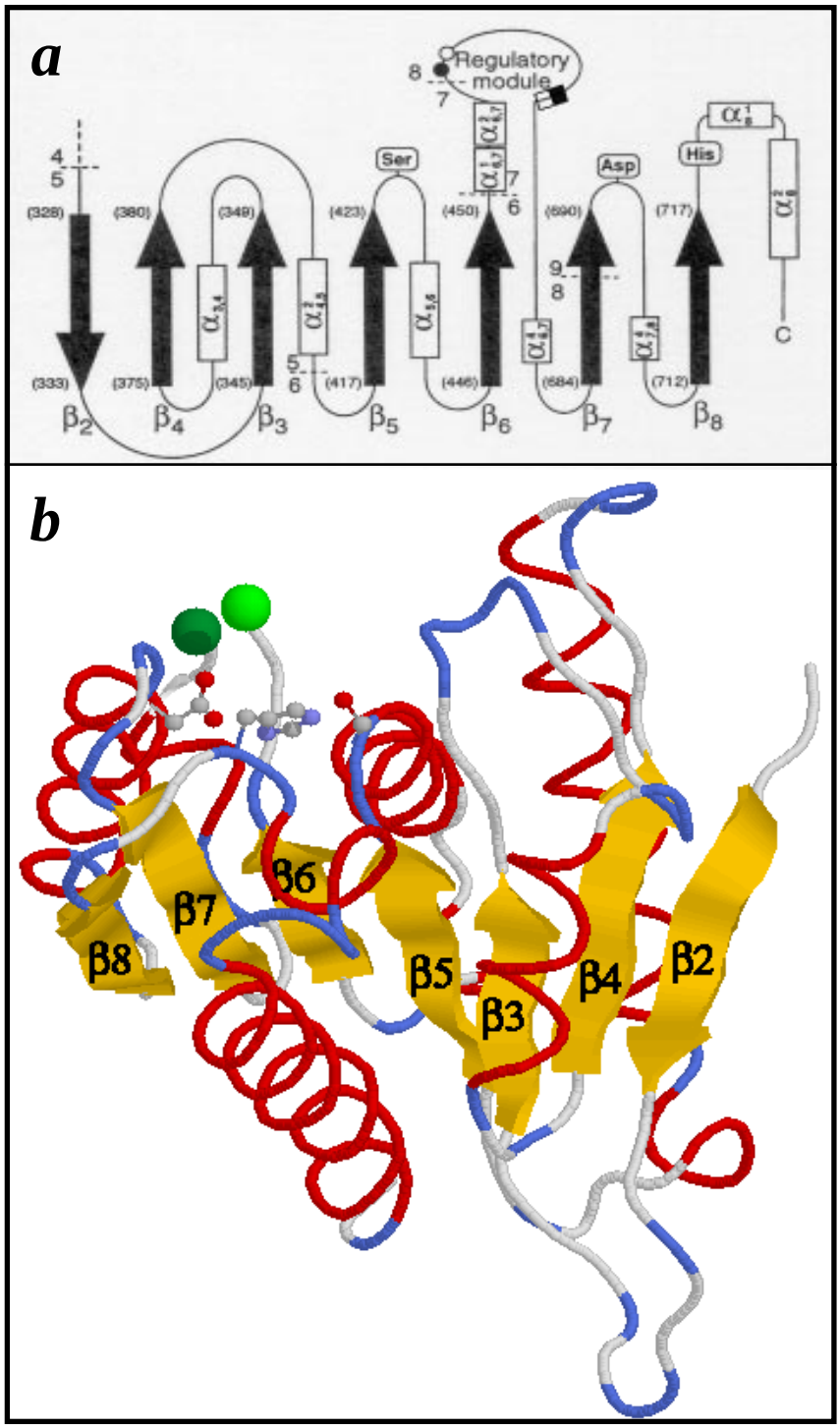


Figure 9;
a) Schematic representation of the catalytic domain of HSL. Exon limits are indicated with dashed lines and the corresponding exon numbers. Also residue positions are indicated. The three catalytic triad residues are indicated with Ser, Asp and His. The region containing the phosphorylation sites protrudes from the protein. The regulatory site and basal site are shown with circles. The estimated position of the newly discovered phosphorylation site are indicated with squares.
b) Ribbon representation of the model for the catalytic domain of HSL. The strands of the central β sheet are numbered accordingly to the enzymes of the carboxylesterase B family. The residues of the catalytic triad are shown in ball and stick representation . The model does not include a vast area located immediately behind the catalytic triad, inserted in the primary sequence between β strands 6 and 7, that constitues a regulatory module. The N- and C-terminal residues of this regulatory module are indicated by a light green and dark green sphere, respectively. (kindly provided by J. A. Contreras and C. Holm)

domain and is located at approximately position 499 to 653 of the rat HSL sequence. Digestion of HSL labeled at site 2, however, did not generate a phosphorylated domain. The label was recovered in a small phosphopeptide suggesting that phosphorylation at site 2 induces a conformational change rendering the regulatory domain susceptible to proteolysis (Smith *et al.* 1996).

When HSL is digested with low concentrations of trypsin, resulting in a loss of HSL activity against lipids, an approximately 11 kDa polypeptide can be detected. This polypeptide amino terminal corresponds to residue 658 and probably runs, consistently with its 11 kDa mass, to the carboxyl end of the HSL protein at residue 768. Furthermore, contributing to its possible role as a lipid-binding region, it contains a hydrophobic region from residue 735 to 742 (FLSLAALC) which could be a possible lipid-binding site (Smith *et al.* 1996).

All together, taking into account the total mass of 84 kDa, the last region to be distinguished is the 35 kDa amino-terminal region. The role of this region remains to be elucidated but possible roles could be in the (regulating) interaction with other proteins, like perilipin.

Summarizing, four domains can be distinguished (***Figure 10***); an amino-terminal domain (approx. residue 1-330), a catalytic domain containing the active site (333 to 499), a regulatory domain containing the two phosphorylation sites (between 499 and 658) and a possible lipid-binding domain (658 and further). Parts of the catalytic triad, however, are located in this last region arguing against a role of this last domain in lipid-binding because with limited tryptic digestion, lipid binding is lost but catalytic activity is retained. This pleads for a role for the amino-terminal domain in lipid interaction as suggested by Østerlund *et al.* (1996).

It should be noted that human HSL is larger then that of rat (88 kDa and 84 kDa respectively) and that of mouse and guinea-pig

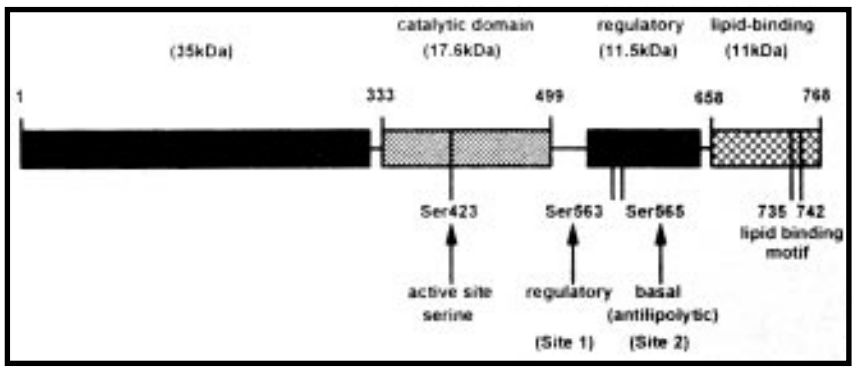


Figure 10;
Proposed domain structure of hormone sensitive lipase. Four regions can be distinguished: a 35 kDa amino terminal domain, a 17.6 kDa catalytic domain, a 11.5 kDa regulatory domain containing two phosphorylation sites, and a 11 kDa putative lipid binding domain containing the newly discovered phosphorylation sites (not indicated). (Adapted from Smith et al. 1996)

smaller (both 82 kDa)(Holm *et al.* 1989). This could implicate some differences between the structure of the various HSL species. The regulation of human HSL, however, appears to be analogous to that of rat HSL (Khoo *et al.* 1974) suggesting that the general structure is conserved.

5 Phosphorylation of hormone sensitive lipase

Initial peptide mapping studies have shown that HSL possesses two phosphorylation sites. Site 1 is called the regulatory site and is thought to have a role in the activation of HSL. This site is phosphorylated by the cyclic AMP-dependent protein kinase and is located at position 563 in the rat HSL sequence (Strålfors *et al.* 1983, 1984, Garton *et al.* 1988) (***Figure 11***). This kinase is hormonally controlled (Strålfors *et al.* 1983, 1984). The hormonal regulation seems to involve a widely used signal transduction mechanism i.e. the cyclic AMP pathway. Binding of the hormone activates adenylate cyclase (AC), which results in increased levels of cyclic AMP. Cyclic AMP on its turn can activate the cyclic AMP dependent protein kinase (also known as protein kinase A or PKA) which phosphorylates site 1 of HSL. Evidence for the role of cyclic AMP in HSL regulation is provided by Salimath *et al.* (1987). They have shown that capsaicin inhibits Ca^{2+} and calmodulin dependent cyclic AMP phosphodiesterase, thereby causing a raise in cAMP levels and subsequent HSL activation. The dephosphorylation, however, does not seem to be hormonally controlled.

Site 2 is called the basal site (Strålfors *et al.* 1984). This site is phosphorylated by three protein kinases, Ca^{2+}/calmodulin-dependent kinase, glycogen synthase kinase-4 (Olsson *et al.* 1986) and

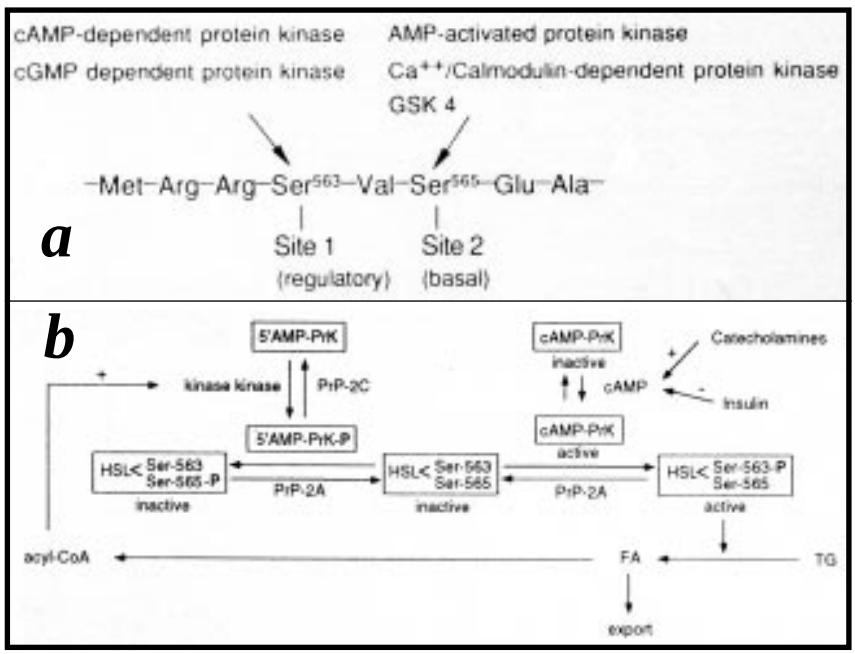


Figure 11;
a) Phosphorylation of the regulatory and basal site. The kinases known to phosphorylate either site in vitro are indicated. (Adapted from Yeaman et al. 1992) b) Hypothesis regarding the short-term regulation of HSL. HSL first has to be dephosphorylated at the basal site, and subsequently phosphorylated at the regulatory site to be active. Lipolytic agents influence the phosphorylation of the regulatory site by modulating the cyclic AMP levels. A possible negative feedback loop of acyl-CoA is also indicated. (Adapted from Holm et al. 1994)

AMP-activated protein kinase (Garton *et al.* 1989), and is located at position 565 in the rat HSL sequence (Strålfors *et al.* 1984, Garton *et al.* 1988, 1989) (***Figure 11***). Phosphorylation on site 2 does not seem to have a direct effect on HSL activity (Garton *et al.* 1989). Interestingly, though, phosphorylation of these two phosphorylation sites is mutually exclusive (Garton *et al.* 1989, 1990). This means that site 2 can possess an anti-lipolytic effect as phosphorylation of site 2 prevents phosphorylation of site 1 and maybe thereby the activation of HSL. The phosphorylation of site 1 is drastically increased by lipolytic agents, whereas lipolytic agents seem to have no effect on the phosphorylation of site 2 (Strålfors *et al.* 1984, 1989).

In contrast to phosphorylation also dephosphorylation by phosphatases can play an important role in HSL regulation. There is evidence that phosphatases are involved in the anti-lipolytic effect of insulin (Butcher *et al.* 1966, Manganiello *et al.* 1973, Wong *et al.* 1981, Elks *et al.* 1983, Londos *et al.* 1985, Strålfors *et al.* 1989). Insulin would activate a cyclic AMP phosphodiesterase (Manganiello *et al.* 1973, Elks *et al.* 1983) which would result in a lower intracellular concentration of cyclic AMP and thereby inhibit cyclic AMP dependent protein kinase. However, there is evidence that insulin can also have a cyclic AMP independent effect on HSL activity. It has been suggested that this would involve the activation of a protein phosphatase (Londos *et al.* 1985, Strålfors *et al.* 1989).

Several commonly known phosphatases are capable of dephosphorylating HSL. Protein phosphatases 1, 2A and 2C are able to do so, although not all to the same extent (Olsson *et al.* 1987). HSL is a good substrate for protein phosphatases 2A and 2C, but not for phosphatase 1. Interestingly, all three phosphatases show a preference for site 2 (the basal site).

A model for the short-term regulation is presented in ***Figure 11b*** (Holm *et al.* 1994). HSL is phosphorylated on residue 563 by PKA resulting in active HSL. The activity of PKA is depenedent on cyclic AMP which levels are modulated by lipolytic hormones. Lipolytic hormones like catecholamines induce an increase in cyclic AMP levels by activating AC. Anti-lipolytic hormones like insulin induce a decrease in the cyclic AMP levels probably by the activation of a cyclic AMP phosphodiesterase. Phosphorylation on residue 565 inhibits phosphorylation on residue 563 and thereby pre-

vents HSL activation, and is phosphorylated by AMP activated protein kinase. This kinase is activated by phosphorylation by a kinase kinase. FFA generated in the breakdown of TAG by HSL are converted into acyl-CoA and it is suggested that acyl-CoA activates this kinase kinase and thereby inhibits its own formation by inactivating HSL.

However, reversible phosphorylation does not seem to be the only mechanism of HSL regulation as the activity of HSL against DAG is not influenced by phosphorylation of HSL (Fredrikson *et al.* 1981). In addition, as discussed later, HSL protein itself is downregulated in lipid laden macrophages (Jepson *et al.* 1996), suggesting there is additional regulation at the transcriptional or translational level.

Recently, Anthonsen *et al.* (1998) found two additional phosphorylation sites in rat HSL (***Figure 12***). They have shown that HSL is phosphorylated at residues 659 and 660 upon treatment of adipocytes, mutated at residues 563 and 565, with protein kinase A. They also showed that site directed mutagenesis of residue 563 or 563,565 did not abolish HSL activity. Site directed mutagenesis of residue 565 did increase HSL activity slightly, which could be expected, as removal of this phosphorylation site lifts its inhibiting effect on the phosphorylation of residue 563. In addition, mutation of both residue 659 and 660 was necessary to abolish HSL activity. This suggests that these new phosphorylation sites are the major sites for HSL regulation, even more as has been shown that phosphorylation of the regulatory site (563) is not sufficient for activation of HSL.

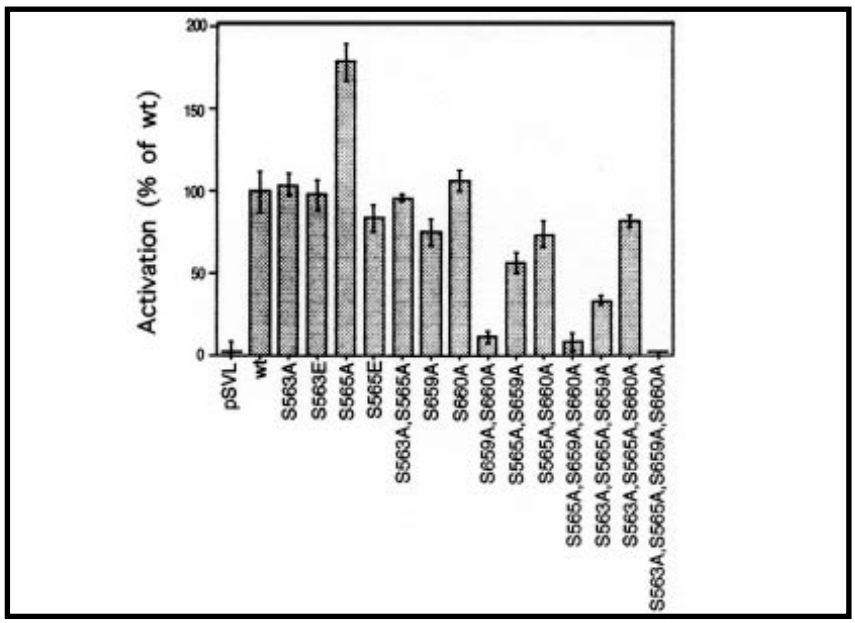


Figure 12;
Activation of HSL mutated at residue 563, 565, 659 and/or 660. Cells transfected with expression vectors encoding HSL (mutated or not). Homogenates were treated with PKA and the activity of HSL measured. Data is indicated relative to non-phosphorylated HSL. Site directed mutagenesis of residue 563 has no effect on HSL activity. Mutation of residue 565 results in an increase in activity due to the loss of inhibition on phosphorylation of residue 563. Mutation of both residue 659 and 660 abolishes HSL activity. (Adapted from Anthonsen et al. 1998)

6 Translocation

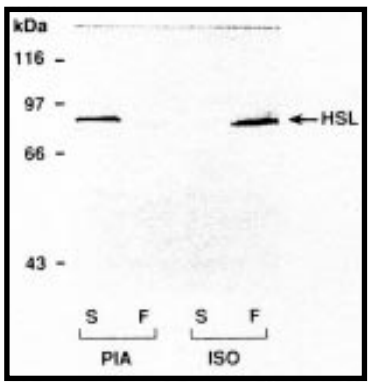
A discrepancy was found between HSL in adipose tissues which was activated maximally by lipolytic agents and *in vitro* phosphorylation of HSL by cyclic AMP-dependent protein kinase. In the first case a 50 to 100 fold increase in HSL activity was observed (Nilsson *et al.* 1980) whereas in the second case only a 2 to 3 fold increase of HSL activity could be seen (Fredrikson *et al.* 1981, Cook *et al.* 1982, Strålfors *et al.* 1983). This indicates that phosphorylation alone is not enough to fully activate HSL.

Several theories have been proposed to explain this. It could be due to a difference in the mode of presentation of the substrate to the enzyme (Belfrage *et al.* 1984) or because the basal site is already (partly) phosphorylated in the purified enzyme used in *in vitro* assays, especially because Garton *et al.* (1988) have shown that the purified HSL is partly phosphorylated. This could prevent further phosphorylation of HSL, as phosphorylation of the regulatory and basal site is mutually exclusive (Garton *et al.* 1989).

A plausible explanation is that upon activation HSL translocates from the cytoplasm to the fat globule. So phosphorylation not only activates HSL but also targets it to the TAG substrate. It was found that upon lipolytic activation the HSL activity in the aqueous fraction (cytosol) decreases whereas the activity in the fat fraction is increased (Hirsch *et al.* 1984, Egan *et al.* 1990, Greenberg *et al.* 1991). Egan *et al.* (1992) provided further evidence for this theory. They showed, with western blotting and a polyclonal antiserum against HSL, that in non-lipolytically stimulated cells almost all the HSL was present in the supernatant and almost non in the fat cake. When the cells were lipolytically stimulated with isoproterenol, all HSL was associated with the fat cake (***Figure 13***). In both cases, the membrane fraction did not contain any HSL. The fact that the translocation is quantitative suggests that the amount of HSL is a rate limiting factor in lipolysis. The role of phosphorylation in the translocation of HSL has been evidenced by Hirsch *et al.* (1984). They showed that addition of 8-Br-cAMP, mimicking an elevation of cyclic AMP levels, was able to induce the translocation of the enzyme.

In addition a fat droplet associated protein has been identified

Figure 13;
Western blotting of adipocyte fractions with antiserum against HSL. A clear translocation of HSL activity from the supernatant (S, the cytoplasmatic fraction) to the fat cake (F) upon stimulation with isoproterenol (ISO). (Adapted from Egan et al. 1992)



whose phosphorylation and dephosphorylation parallels that of HSL (Egan *et al.* 1989, Greenberg *et al.* 1991). This protein, named perilipin, may localize HSL to the fat droplet by binding to it or at least participate in some way in lipolysis.

As a result of these findings Egan *et al.* (1992) postulated the idea that HSL is constitutively active, phosphorylated or not, but that it is just inaccessible to the cellular substrate. This is supported by the findings of Strålfors *et al.* (1977) who showed that the difference in HSL activity of stimulated and unstimulated cells disappeared upon sonication of the cells. The role of the phosphorylation would be to increase the access of HSL to the substrate by translocating the HSL to the lipid droplet. In addition, HSL is not freely soluble in the cytosol. HSL could be bound to a cytosolic factor and be released upon phosphorylation (Londos *et al.* 1995). Furthermore, phosphorylation could enhance the interaction between HSL and the lipid droplet due to, for instance, a change in conformation of HSL.

7 Perilipins

Perilipins are the most abundantly labeled proteins after lipolytic activation in adipocytes (Egan *et al.* 1990, Greenberg *et al.* 1991, 1993). Most interestingly, their response to lipolytic agents, i.e. phosphorylation, parallels that of HSL. The perilipin gene gives rise to multiple isoforms by alternative RNA splicing. They differ in their C-terminal regions, whereas the N-terminal regions are identical. Both perilipin A and B contain three phosphorylation sites in their shared region. Perilipin A has three additional phosphorylation sites in its unique C-terminal region (Greenberg *et al.* 1993) (***Figure 14***).

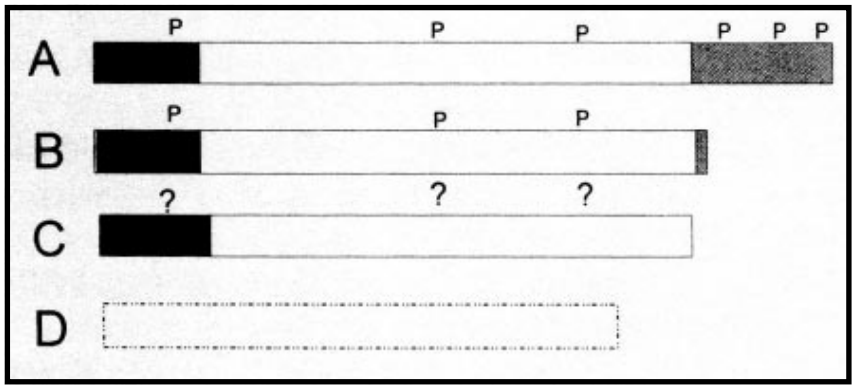


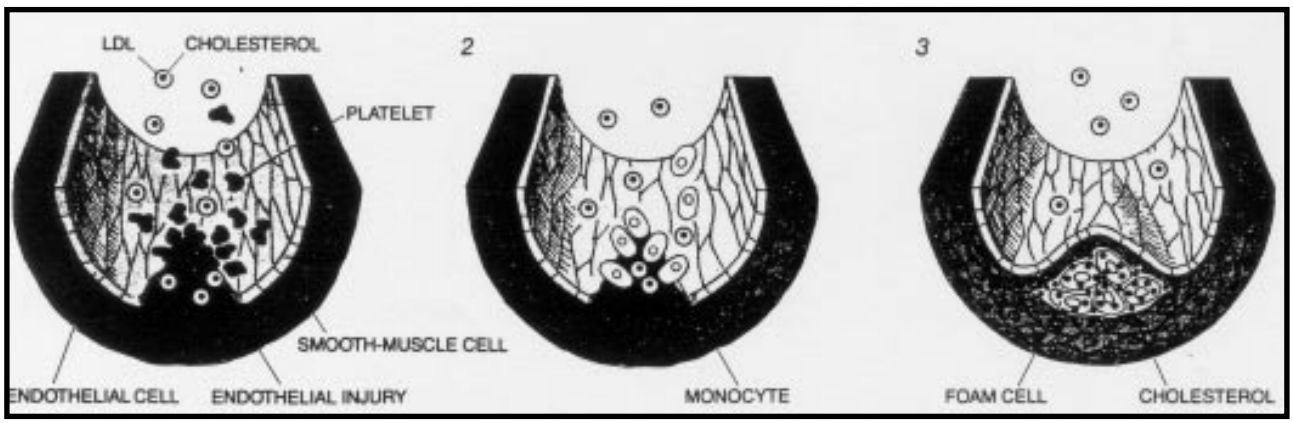
Figure 14;
Schematic view of perilipins isoforms. Murine adipocytes express four species, but only the A and B isoforms are detected in rat fat cells. A and B differ only in their C-terminal regions. P indicates PKA phosphorylation sites. The mRNA for C is relatively scarce in adipocytes and abundant in steroidogenic cells. The N-terminal region of C is similar but not identical to the N-terminal region of A and B. D is an abundant mRNA in adipocytes, however, no corresponding protein has been identified. (Adapted from Londos et al. 1995)

Beside adipose tissue, perilipins can, like HSL, also be found in steroid-hormone producing cells (Yeaman 1990, Londos *et al.* 1995). In these cells an additional perilipin could be found, designated perilipin C (***Figure 14***). No phosphorylation sites are know yet for this perilipin (Londos *et al.* 1995). Although no common lipid binding motifs can be found in perilipins, they directly associate with the first formed lipid depositions. In addition, when undifferentiated 3T3-L1 cells are transfected with perilipin A encoding constructs, numerous small lipid droplets are formed suggesting that perilipin might serve as a formation site for lipid droplets (Londos *et al.* 1995). This possible role of perilipin in lipid packaging seems to be in contradiction with the suggested role of perilipin in HSL mediated lipid hydrolysis. Londos *et al.* (1995) postulate the possible explanation that non-phosphorylated perilipin might serve a role in lipid packaging, and that phosphorylated perilipin has a role in tethering HSL to the fat droplet.

8 Artherosclerosis

Atherosclerosis is an arterial decease in which the formation of fatty plaques in the blood vessels results in thickening, loss of elasticity and in the end obstruction of the vessel (***Figure 15***). Macrophages which are overloaded with cholesterol esters form a major source of foam cells, lipid laden cells that form the fatty plaques. Macrophages normally reside in the arterial wall or circulate in the blood stream and penetrate sites of damage (Steinberg *et al.* 1987). Here macrophages accumulate cholesterol esters by the uptake of lipoproteins, like LDL which is the major lipoprotein for cholesterol transport. Internalized cholesterol esters are hydrolyzed in the lysosomes by an acid hydrolase (Small *et al.* 1989a, 1990), and the resulting cholesterol is released into the cytoplasm (**Figure 16**). In the cytoplasm the cholesterol is either esterified by acyl-CoA:cholesterol acyl transferase (ACAT) to cholesterol esters (Brown *et al.* 1980), or released from the cell to a HDL vesicle, if present. Stored cholesterol esters can subsequently be hydrolyzed by a neutral cholesterol ester hydrolase. So cholesterol esters in macrophages are continuously hydrolyzed and re-esterified by HSL and ACAT respectively (Brown *et al.* 1980). The re-esterification, however, uses ATP making the cycling of cholesterol esters an eneregy waisting process. When an acceptor for the free cholesterol is present (like HDL) there is a net hydrolysis of cholesterol esters. The hydrolyzing reactions is, however, not increased. It is the esterification reaction that decreases. The reduced availability of substrate for ACAT might be the reason for this, but it is also suggested that the reduction of the free cholesterol pool decreases the catalytic activity of ACAT (Brown *et al.* 1975, Goldstein *et al.*

Figure 15;
Formation of atherosclerotic lesions in the blood vessel. Endothelial damage initiates the formation of atherosclerotic plaques. The damaged endothelium becomes leaky and is penetrated by blood platelets and LDP particles (1). Next, the smooth muscle cells start to multiply and migrate into the damaged area (2). At the same time macrophages enter the damaged area and start to ingest and degrade LDL and transform into foam cells. When the internalized cholesterol accumulates (because esterification exceeds the hydrolysis) the accumulated cholesterol, cells and debris form an atherosclerotic lesion (3). (Source unknown)



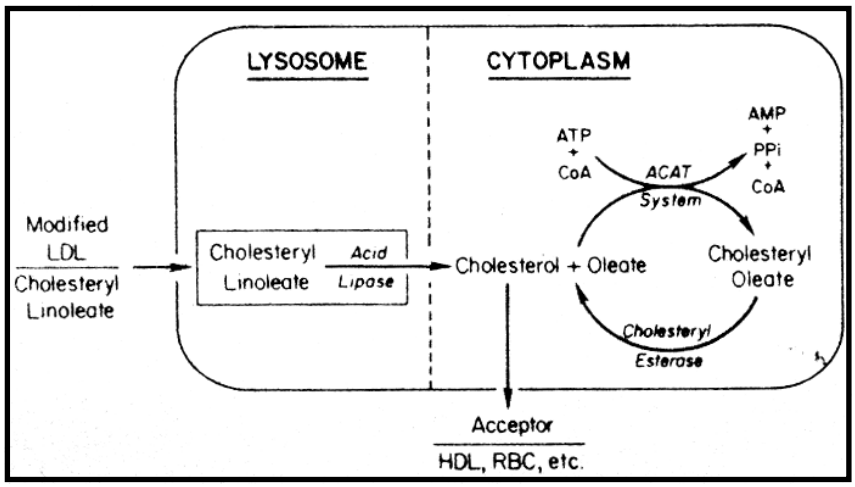


Figure 16;
Model illustrating the cholesterol ester cycle. Cholesterol taken up from LDL is hydrolyzed in the lysosome and the free cholesterol is secreted into the cytoplasm. Here it is esterified by ACAT if not loaded onto an external exceptor like HDL. Cholesterol esters can subsequently be hydrolyzed by HSL to free cholesterol. (Adapted from Brown et al. 1980)

1977).

It is evidenced that the cycling of cholesterol esters, besides *in vitro*, also occurs *in vivo* atherosclerotic foam cells (St. Clair *et al.* 1976). When the rate of re-esterification exceeds the rate of hydrolysis, accumulation of cholesterol esters will take place, and foam cells will develop (Steinberg 1987). Evidence for HSL being responsible for the neutral cholesterol ester hydrolyzing activity was provided in immunological studies (Small *et al.* 1989a). Antibodies against HSL inhibit the neutral cholesterol ester hydrolase activity completely and immunoprecipitate a 84 kDa protein from macrophage extract.

Yeaman *et al.* (1994) have shown that the activity of HSL is greatly reduced to totally lost in foam cells. Additionally, Jepson *et al.* (1996) have shown, with western blotting, that the decrease in HSL activity is caused by a decrease in HSL protein and their results suggest that the level of HSL protein is directly related to the amount of intracellular sterol esters. The reason for the downregulation of HSL protein is not yet fully understood. This does, however, suggest that HSL has an important role in the regulation of this cholesterol ester cycle, and thus in foam cell development.

Beside HSL, also ACAT seems to have a role in the regulation of this cycle. Lipid laden cells show an increased ACAT activity (Brown *et al.* 1980, Tabas *et al.* 1987, Xu *et al.* 1991) and thereby contribute to the cholesterol ester accumulation. Also the acid hydrolase in the lysosome can play a role in the accumulation cholesterol esters. An increase in the acid hydrolase activity could result in increased levels of free cholesterol in the cytoplasm. This increase in free cholesterol results in an increase in ACAT activity and subsequently in an increase in cholesterol ester levels. There is,

however, at this moment no direct evidence for this.

Atherosclerosis is, however, far more complex than stated here. Several other metabolic alterations have to occur to develop atherosclerotic lesions, and genetic and environmental factors can modify one's susceptibility for this disease. A more thorough discussion about these additional factors necessary to develop atherosclerosis is beyond the scope of this thesis.

9 Model

In this chapter it is tried to compose a model of the regulation of HSL (***Figure 17***). Upon a lipolytic signal the receptor activates adenylate cyclase. This results in an increase in the cyclic AMP levels, resulting in the activation of PKA. This is then capable of phosphorylating HSL at residue 563. This requires HSL to be dephosphorylated at residue 565 by a protein phosphatase. PKA also phosphorylates HSL at residues 659 and 660. At the same time perilipin is phosphorylated. The phosphorylated HSL is now active, possibly due to a conformational change exposing the active site and/or enhancing its availability to the substrate. In addition, HSL translocates from the cytoplasm to the lipid droplet, perhaps aided by perilipin. This activation of HSL can be fine-tuned at several levels. Insulin is able to decrease HSL activity, by activating a cyclic AMP phosphodiesterase resulting in a decrease in cyclic AMP levels and in this way inhibiting PKA, and/or by activating a phos-

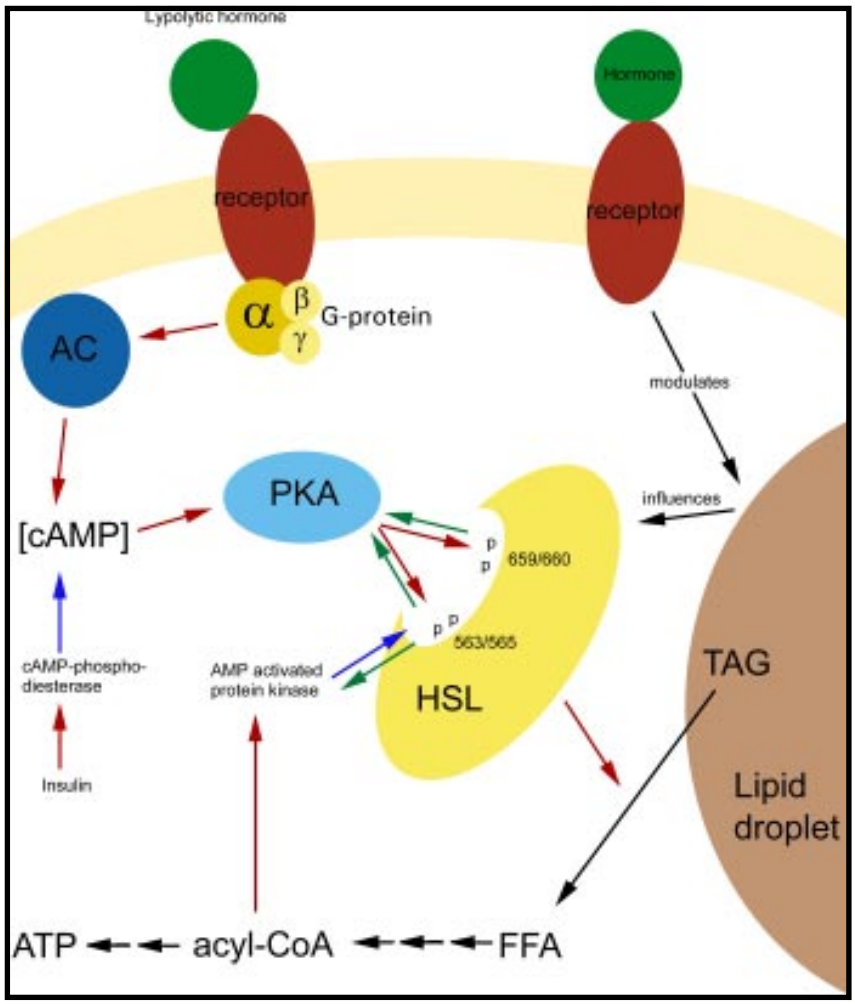


Figure 17;
Model for HSL regulation. Red arrows indicate an activation, blue arrows an inhibition and black arrows indicates another kind of relation. The green arrows represent protein kinase activity.

phatase that dephosphorylates HSL at residue 563. In addition, an intermediate in the breakdown of FFA, acyl-CoA, is thought to be able to provide a negative feedback by enhancing the phosphorylation of residue 565. As stated in chapter 3, also the lipid droplet composition can influence HSL activity. The composition of the droplet can be altered in response to external signals, like hormones. Another possible important factor in the regulation is the HSL transcription and translation. However, nothing is known yet about the regulation of the HSL gene.

10 Concluding remarks

The use of energy is tightly regulated in every living organism. Correct regulation of uptake, storage and release of energy sources is of vital importance for survival. It is not strange that a tremendous amount of research has been done to reveal how the energy metabolism functions. The knowledge of the catalytic pathways of sugars and fatty acids is very detailed. So is the knowledge about the regulation of carbohydrate sources. However, until recently, there was a severe lack of knowledge regarding the regulation of the lipid metabolism. The enzymatic pathways are almost clear, but their regulation is, for a great part, still a mystery.

A key role in the breakdown of stored lipids, and thereby in the regulation of energy availability, is played by a rate limiting enzyme. This enzyme, called hormone sensitive lipase or HSL, was the focus of this thesis. This thesis tried to present an overview of all the aspects of HSL presently known. It will be clear that our understanding of HSL, and related subjects, although growing, is still marginal. Not alone because of the added complexity by HSL's dual role, hydrolysis of lipids and also hydrolysis of cholesterol esters.

HSL is regulated by reversible phosphorylation which is not seen with any other known lipase. It can by phosphorylated at 4 residues; 563, 565, 659 and 660. Phosphorylation of residue 565 blocks the phosphorylation of residue 563. Phosphorylation at the regulatory site (563) was thought to be the main switch in the activation of HSL. Anthonsen *et al.* (1998) however have shown that phosphorylation of the regulatory site is only partly responsible for the activation of HSL as site directed mutagenesis results in only a small decrease in activity. They show that phosphorylation of both residue 659 and 660 is necessary for the activation of HSL, as mutagenesis of both residues abolishes HSL activity. The question that arises is, what is the role of residues 563 and 565? The role of phosphorylation on residue 565 will clearly be the fine regulation of phosphorylation on residue 563. One could speculate that phosphorylation of 563 could have a role in the translocation of HSL to the lipid droplet. Another possibility is that it induces a conformational change that enhances the availability of the enzyme to the

substrate. The role of phosphorylation in the translocation can easily be investigated by a combination of site directed mutagenesis, as performed by Anthonsen *et al.* (1998), and HSL localization studies, as performed by Egan *et al.* (1992).

The possible role of perilipins in the translocation of HSL also deserves further investigation. The parallel phosphorylation of perilipins with HSL in response to lipolytic agents suggests a relation with the regulation of HSL in some way. Also the affinity of perilipins for lipid depositions and its ability to induce the formation of lipid droplets in non-adipose cells (Londos *et al.* 1995) suggest a role in lipid metabolism. And also the equal distribution in tissues of perilipin, compared to HSL, adds to this possible role. However, no direct relation has been evidenced so far. Greenberg *et al.* (1991) proposed an interesting possibility, however. They suggest that perilipin might have a gating and docking function for HSL, binding HSL to the fat droplet and presenting TAG to HSL.

The HSL gene is regulated by a TATA-less promoter. The factors regulating this promoter are still largely unknown. Binding regions for the Sp1 transcription factors have been indicated, which are commonly seen in household genes. *Li et al.* (1994) mentioned the presence of gen regulatory elements in the 5' region, and suggests a role for them in the tissue specificity of HSL. In addition, Goldstein *et al.* (1990) have reported the presence of a sterol regulatory element. There are at this moment virtually no reports of other regulatory sequences or enhancer regions. Now that the basal promoter structure has been identified, our understanding of the regulation of the HSL promoter will rapidly evolve.

The several distinguishable domains of HSL seem to be located within their own exon or exons (***Figure 4***). This strongly suggests that HSL is a mosaic protein.

Whereas the primary structure and the gene structure do not resemble that of any other lipase or cholesterol ester hydrolase, the secondary structure presents homology with some other enzymes (Contreras *et al.* 1996). The HSL structure consists of various α helixes and β sheets folded in a so called α/β hydrolase fold. As said, HSL consists of several seperate domains. Smith et al. suggested a structure of HSL with four functional domains; a catalytic domain, a regulatory domain, a lipid binding domain and an amino terminal domain with no clear function. The modeling studies of

Contreras *et al.* (1996), however, indicate that this four domain structure is possibly not valid. The proposed catalytic, regulatory and lipid binding domains fold into one structure (***Figure 9a***). The regulatory domain is encompassed between the regions composing the catalytic triad. The proposed lipid binding domain possesses two of the three residues that form the catalytic triad (Asp and His). When, as was suggested by Smith *et al.* (1996), digestion of HSL with a low concentration of trypsin would result in the loss of the proposed lipid binding domain, it would also result in the loss of a functional catalytic triad, and thereby probably in loss of HSL activity. Remarkably, however, Smith *et al.* (1996) showed that HSL activity against a water soluble substrate was maintained and their data do show that the region from residue 658 and up is removed upon tryptic digestion. The question that arises is how can HSL have hydrolyzing activity when it doesn't possess a functional catalytic triad? This is a very important question for which there is no answer yet. Østerlund *et al.* (1996), however, ascribe the lipid binding properties of HSL to the amino terminal domain and propose a two domain structure of HSL. These seemingly contradictory data clearly needs further investigation.

This thesis tried to provide a general overview of the, though limited, knowledge of the function, structure and regulation of HSL. For many years scientists were focussed on the carbohydrate metabolism. Now, one can see a clear increase in the interest in lipid metabolism, possibly fed by rapidly growing health issues like obesity and atherosclerosis. Their is a whole new unrevealed field in front of us, waiting to be explored.

Have a nice journey!

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I'm almost past another milestone. I wonder were the road will lead me.

The future is a convenient place for dreams
ANATOLE FRANCE (1844-1924)

Make wisdom your provision for the journey from youth to old age, for it is a more certain support than all other possessions.
BIAS (570 B.C.)