FEBS Letters 396 (1996) 90–94 FEBS 17682

Evidence for a multi-domain structure for hormone-sensitive lipase

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Received 17 September 1996

Abstract Hormone-sensitive lipase (HSL) is a multi-functional enzyme involved in several aspects of lipid metabolism. Limited tryptic digestion of HSL led to selective loss of activity against lipid substrates but not against the water-soluble substrate, *p*-nitrophenyl butyrate. Following labelling of the active site of HSL with either [^{3}H]di-isopropylfluorophosphate or [^{14}C]orlistat, tryptic digestion of HSL generated a stable radiolabelled domain of molecular mass approx. 17.6 kDa, consistent with this representing a catalytic domain of HSL capable of hydrolysing water-soluble but not lipid substrates. Following phosphorylation of HSL by cyclic AMP-dependent protein kinase, limited tryptic digestion produced a stable phosphorylated domain of molecular mass 11.5 kDa. Based on these experimental data a model for a domain structure of HSL is proposed.

Key words: Hormone-sensitive lipase; Domain structure; Active site; Lipolysis

1. Introduction

Hormone-sensitive lipase (HSL) is the enzyme responsible for catalysing the initial steps of adipocyte lipolysis, and in addition has other diverse functions in lipid metabolism in a variety of tissues [1]. The enzyme, and in particular its regulation by reversible phosphorylation, has been studied extensively since it was initially identified over 30 years ago [2], but it is only relatively recently that details of the structure of the enzyme molecule have started to become available, and as yet very little is known about the molecular mechanism whereby HSL is activated by phosphorylation.

The amino acid sequences of rat HSL, comprising 768 amino acid residues [3,4], and human HSL, comprising 786 amino acid residues [4] have been deduced from cDNA sequencing. Although it was reported initially that the protein sequence of HSL shows no similarity with any other sequenced eukaryotic protein [3], distant similarity with lipoprotein lipase has now been reported [5]. Furthermore, it has been demonstrated that HSL shares significant similarity (approx. 40% identity) with a lipase from the antarctic bacterium *Moraxella* TA144 [4,5] and with several other bacterial proteins including an esterase and two acetyl hydrolases [5,6]. This similarity is in the region including the putative active site serine at 423 and is approx. 70 residues amino-terminal to the active site. Notwithstanding this recent information, the deduced protein sequence of HSL reveals very little information regarding the structure-function relationship of the enzyme. It does, however, allow the location of both phosphorylation sites to be assigned: site 1, the regulatory phosphorylation site is at Ser-563 in the rat HSL sequence, while site 2, the basal phosphorylation site, is Ser-565 [7,8]. In addition, on the basis of similarity with other lipases and esterases, a GXSXG active-site motif was identified at residues 421–425 (GDSAG) and modification of Ser-423, followed by expression of HSL in COS cells has provided direct evidence that this serine residue is essential for catalytic activity [9].

HSL has a relatively broad substrate specificity, being not only capable of hydrolysing a variety of lipid substrates, but also having activity against the water-soluble substrate *p*-nitrophenyl butyrate (PNPB) [10]. Limited tryptic digestion of bovine HSL reduces the activity of the enzyme against lipid substrates to a greater extent than against PNPB, indicating the possible presence of a distinct lipid-binding site essential for activity against lipid substrates, consistent with data for other mammalian lipases [10]. We report here evidence for a multi-domain structure of HSL which will serve as a rational model on which to base future studies of the structure and regulation of this key enzyme.

2. Materials and methods

2.1. Materials

Di[1,3-^{3}H]isopropylfluorophosphate ([^{3}H]DFP) was obtained from NEN and [γ-^{32}P]ATP from ICN Radiochemicals. Tri[^{3}H]oleoylglycerol and 1(3)-mono[^{3}H]oleoyl-2-oleylglycerol (MOME) were kindly supplied by Prof. P. Belfrage (University of Lund, Sweden). [^{14}C]Orlistat (formerly known as tetrahydrolipstatin) was a generous gift from Prof. A. Fischli (Hoffmann La Roche, Basel, Switzerland). Trypsin (L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK)-treated) was from Worthington. Soybean trypsin inhibitor (type I-S) and PNPB were from Sigma. Sources of other materials were as described in [11].

2.2. Enzyme preparations and assays

The catalytic subunit of cyclic AMP-dependent protein kinase was purified to approx. 95% purity from bovine heart. Preparations of AMP-activated protein kinase from rat liver were generously provided by the laboratory of Prof. D.G. Hardie (University of Dundee, UK). HSL was prepared from bovine adipose tissue essentially as described previously [11] except that the final purification step (heparin-Sepharose) was replaced by chromatography on a Mono S FPLC column (Pharmacia, Uppsala, Sweden), followed by dialysis against buffer A (10 mM HEPES, pH 7.0, containing 0.1 mM benzamidine, 50% (w/v) glycerol, 1 mM dithiothreitol, 5 μg/ml leupeptin, 0.2% (w/v) $C_{13}E_{12}$). The enzyme was stored at −80°C. HSL was assayed against 0.5 mM tri[^{3}H]oleoylglycerol in ethanolic suspension based on the method in [12]. HSL activity against 1-[^{3}H]MOME, a diacylglycerol analogue, was carried out in the presence of phospholipids as in [13]. Assays against the water-soluble substrate, PNPB, were performed essentially as in [14].

2.3. Limited tryptic digestion and labelling of HSL

Limited tryptic digestion of HSL was carried out at pH 7.0 and 25°C. Native HSL was digested at a final concentration of 10 μg/ml protein by incubation for 15 min in the presence of 10 mM HEPES, pH 7.0, containing 10% (v/v) glycerol, 10 mM NaCl, 1 mM dithiothreitol and varying concentrations of trypsin. Digestion was termi-

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PII S0014-5793(96)01076-9

nated by the addition of trypsin inhibitor to 20 μg/ml. Samples were immediately frozen in liquid nitrogen until subsequent assaying or radiolabelling. Samples of native HSL, or HSL which had been digested with trypsin as described above, were subjected to [^{3}H]DFP labelling by adding to [^{3}H]DFP (0.5 μCi/nmol) to a final concentration of 0.175 mM DFP and the samples incubated for 30 min at 30°C. This treatment covalently modifies the enzyme and inhibits the activity of HSL by greater than 95% [15]. Samples were then prepared for SDS-PAGE by precipitation of the protein with 20% (w/v) ice-cold trichloroacetic acid and resuspension in 250 mM Tris-HCl, pH 6.8, containing 20% (w/v) glycerol, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue. HSL was [^{32}P]phosphorylated by incubation for 15 min at 30°C in the presence of 0.1 mM [γ-^{32}P]ATP (1 μCi/nmol) and 5.4 mM $MgCl_2$. Phosphorylation was catalysed by either 1.0 U/ml catalytic subunit of cyclic AMP-dependent protein kinase or 1.4 U/ml AMP-activated protein kinase in the presence of 1.5 mM AMP. [^{32}P]HSL was then subjected to limited tryptic digestion as described above.

2.4. Electroblotting and sequence analysis

SDS-PAGE was performed as in [16] using either 13.5 or 15% (w/v) polyacrylamide resolving gels. Proteins were electroblotted from gels onto polyvinylidine difluoride (PVDF) membranes (Pall Europe Ltd., Portsmouth, UK) for 4 h at 0.3 A using 10 mM CAPS (3-[cyclohexylamino]-1-propanesulphonic acid), pH 11.0 in 10% (v/v) methanol. Transfer was complete as judged by the absence of Coomassie staining of proteins on the original gel. The membranes were stained with 0.1% (w/v) Coomassie Blue in 1% (v/v) acetic acid, 40% (v/v) methanol and destained with 50% (v/v) methanol. The membranes were then either subjected to direct autoradiography or the bands of interest were excised for sequence analysis on Applied Biosystems 470A gas-phase and 477A pulsed liquid-phase protein sequencers using recommended reagents with 120A on-line phenylthiohydantoin (Pth)-amino acid analysers [17]. Data collection and analysis were performed with an Applied Biosystems 900A module calibrated with 25 pmol Pth-amino acid standards.

3. Results and discussion

The water-soluble PNPB is an effective substrate for HSL, with maximum catalytic activity (150 U/mg) against that substrate being approx. 10-fold higher than that against trioleoylglycerol under the assay conditions employed (not shown). Lineweaver-Burk analysis gave a K_m of 0.21 mM for PNPB (a similar value has been reported in [10]) and an apparent K_m of 0.13 mM for trioleoylglycerol.

Limited tryptic digestion of native HSL was found to reduce dramatically the activity of the enzyme against trioleoylglycerol, whilst the activity against PNPB was relatively trypsin-insensitive (Fig. 1A). The differential effect of trypsin on these activities was such that after 15 min of digestion at 25°C with 2 μg/ml trypsin, approx. 75% of the PNPB hydrolase activity of HSL remained, whereas activity against trioleoylglycerol was reduced to less than 10% of the initial value. After digestion with 10 μg/ml, activity against trioleoylglycerol was virtually undetectable whilst that against PNPB was approx. 50% of the initial. Similarly, when the activities against the two different substrates were monitored as a function of time of digestion with a fixed amount (2 μg/ml) of trypsin, the activity against trioleoylglycerol decreased rapidly but the activity against PNPB was effectively retained (not shown). These results suggest that limited tryptic digestion of HSL selectively removes or destroys a region of the molecule responsible for binding lipid substrates, whilst a comparatively stable catalytic region of the enzyme remains intact and is capable of hydrolysing water-soluble substrates.

In order to investigate further this possibility, HSL was digested with the same range of trypsin concentrations and the resultant protein fragments were incubated with [^{3}H]DFP in order to label specifically the active site of any fragments which retain catalytic activity. The labelled fragments were then separated by SDS-PAGE, electroblotted and analysed by direct autoradiography. The resultant autoradiograph showed the presence of several labelled fragments, which,

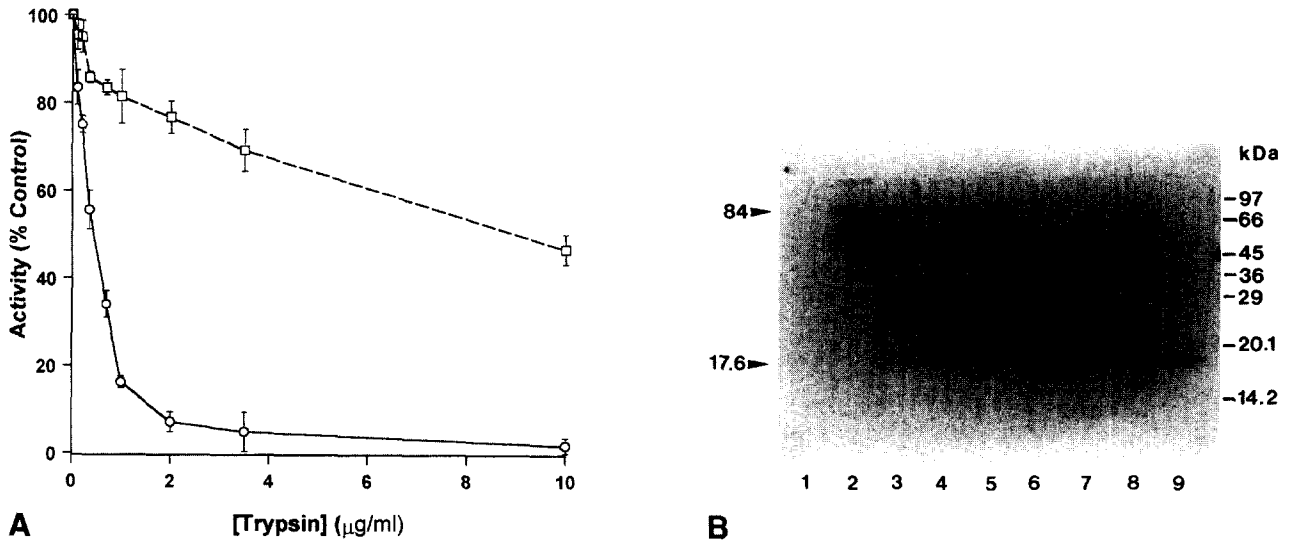

Fig. 1. Limited tryptic digestion of HSL. Purified HSL (at a final concentration of 10 μg/ml HSL enzyme protein) was subjected to proteolytic digestion at pH 7.0 for 15 min at 25°C in the presence of the indicated concentration of trypsin as described in Section 2. Digestion of the protein was terminated by the addition of trypsin inhibitor to 20 μg/ml. (A) Aliquots were then assayed in duplicate against tri[^{3}H]oleoylglycerol (○) or PNPB (□) as described. Results are expressed as a percentage of the activity observed in the absence of trypsin. Each point represents the mean ± S.E.M. for 5 separate experiments. (B) Aliquots were incubated with 0.175 mM [^{3}H]DFP for 30 min at 30°C as described in Section 2. The protein fragments were then recovered by trichloroacetic acid precipitation, separated by SDS-PAGE, electroblotted and the radiolabelled polypeptides analysed by direct autoradiography. Lanes 2–9 each contain approx. 0.5 μg HSL enzyme protein and the indicated concentrations of trypsin: lanes: 1, control, buffer A+10 μg/ml; 2, undigested HSL; 3, 0.1 μg/ml; 4, 0.2 μg/ml; 5, 0.3 μg/ml; 6, 0.6 μg/ml; 7, 1 μg/ml; 8, 2 μg/ml; 9, 10 μg/ml.

with increasing digestion, are converted to the smallest radiolabelled fragment of HSL (17.6 kDa) which is quite stable to tryptic digestion since increasing the final trypsin concentration from 0.6 to 10 μg/ml had little effect on the intensity of this polypeptide band after autoradiography (Fig. 1B). [^{3}H]DFP labelling of the 17.6 kDa fragment occurs under conditions in which activity against lipids has been lost, which indicates that the conformation of the active site of the enzyme must remain stable within this fragment which is able to catalyse the hydrolysis of a water-soluble substrate and to react with DFP even though activity against triacylglycerol has been virtually eliminated. It appears, therefore, that the active site is contained within a relatively stable, putative catalytic domain (17.6 kDa) which may represent the minimum active unit which is required for esterase activity.

Orlistat, formerly known as tetrahydrolipstatin, is a general lipase inhibitor derived from lipstatin, a lipid produced by *Streptomyces toxytricini* [18] and has been shown to inhibit in vitro several mammalian lipases including pancreatic lipase, gastric lipase and bile-salt activated lipase by binding at the active site [19,20]. When HSL was incubated with increasing concentrations of orlistat it was found that 1–10 mM orlistat caused approx. 90% loss of activity against lipid substrates and against PNPB (not shown). When HSL was incubated with [^{14}C]orlistat, the 84 kDa polypeptide became radiolabelled (Fig. 2), and following limited tryptic digestion, the same stable domain of mass 17.6 kDa (as labelled with [^{3}H]DFP) was detected following SDS-PAGE and autoradiography (Fig. 2). Also, when HSL was subjected to limited proteolysis (as in Fig. 1) followed by incubation with [^{14}C]orlistat, the same pattern of radiolabelling fragments was observed (Fig. 2) as with the labelling with [^{3}H]DFP. When HSL was pre-incubated with increasing concentrations of non-radioactive orlistat prior to incubation with [^{3}H]DFP, incorporation of radiolabel into the HSL polypeptide was blocked in a concentration-dependent manner (not shown). All the above observations are consistent with the presence of a single active site susceptible to modification by both DFP

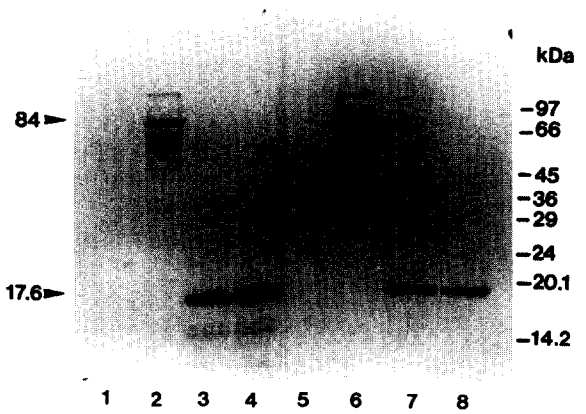

Fig. 2. Covalent modification of HSL with [^{14}C]orlistat. Purified HSL (5 μl containing approx. 0.5 μg HSL enzyme protein) was radiolabelled with [^{14}C]orlistat (0.1 mM) as described in Fig. 1. [^{14}C]HSL was then subjected to limited tryptic digestion (lanes 1–4) for 15 min at 25°C. In comparison, native HSL was subjected to limited tryptic digestion followed by [^{14}C]orlistat labelling of the digestion fragments (lanes 5–8). Radiolabelled fragments were analysed as described in Section 2. Lanes: 1,5, control, buffer A+20 μg/ml trypsin; 2,6, undigested HSL; 3,7, HSL+10 μg/ml trypsin; 4,8, HSL+20 μg/ml trypsin.

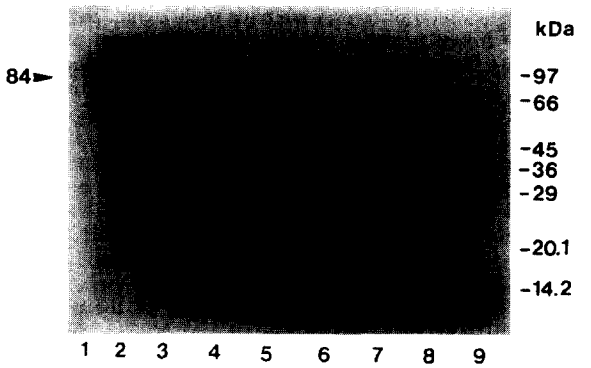

Fig. 3. Limited digestion of [^{32}P]HSL. Purified HSL (2 μl, containing approx. 0.2 μg HSL enzyme protein) was phosphorylated with [γ-^{32}P]ATP at the regulatory site by cyclic AMP-dependent protein kinase, as described in Section 2. [^{32}P]HSL was then subjected to limited tryptic digestion as in Fig. 1. Lanes 2–8 contained HSL plus the indicated amounts of trypsin. (A) Lanes: 1, control, buffer A+20 μg/ml trypsin; 2, undigested HSL; 3, 0.2 μg/ml; 4, 0.4 μg/ml; 5, 2 μg/ml; 6, 4 μg/ml; 7, 10 μg/ml; 8, 15 μg/ml; 9, 20 μg/ml trypsin.

and orlistat, the data in [9] further suggesting that this site of modification is Ser-423.

To obtain a basis for more detailed structural studies of the HSL protein, the 17.6 kDa fragment was subjected to N-terminal sequence analysis after electroblotting. The sequence obtained was: LRPQQAPRSrALVVHIhGGgFVXQXS (amino acid residues shown in lower case are tentative assignments, X denotes no residue assigned). Only one Pth-amino acid residue was found at each cycle confirming the purity of the fragment. Comparison of this sequence with the predicted protein sequence of rat [3] and human HSL [4] reveals that it corresponds to residues 333–358 in both sequences. The HSL sequences show a high level of homology in this region, with, for example, only the N-terminal leucine in the bovine sequence positively identified as being different from rat HSL (which has proline in this position). Based on an apparent mass of 17.6 kDa, the C-terminus of the tryptic fragment would be at approximately residue 498 (calculated using the rat HSL sequence with an average residue weight of 109.4). Since there is a Lys residue at position 499 in rat and human HSL, this is likely to represent the C-terminus of the fragment, although there are other potential tryptic cleavage sites in both rat and human HSL. Bovine HSL used here does indeed contain a Lys or Arg residue at position 499, since Holm et al. [3] obtained a tryptic peptide ALGVMGVQR which commences at position 500. Hence residue 499 is likely to be the C-terminal residue of this 17.6 kDa fragment.

This work makes significant progress towards identifying the active site of HSL by eliminating approx. 80% of the enzyme molecule, and localising its position to within a 17.6 kDa domain. Interestingly, this putative catalytic domain of HSL contains the region exhibiting sequence similarity with the bacterial lipase. Although no direct evidence is available it is predicted that by analogy with the other lipases HSL will have a histidine and aspartic acid residue forming a catalytic triad with the active site serine at position 423. Identification of these additional catalytic residues will require either determination of the three-dimensional structure of HSL (or of the putative catalytic domain) or alternatively site-specific mutagenesis of each histidine or aspartate residue. The 5 histidine

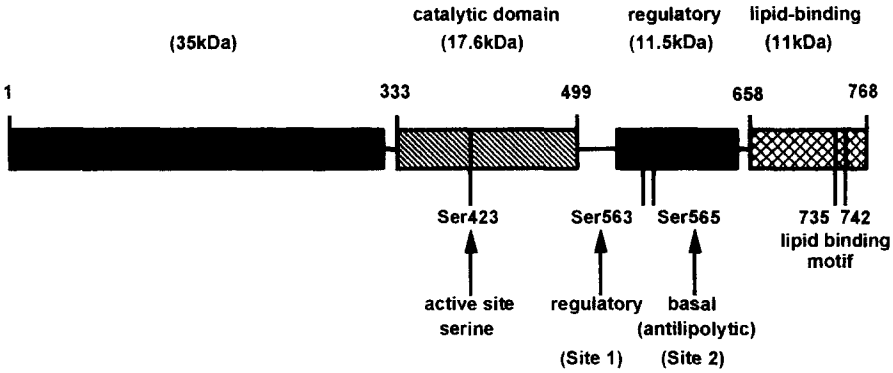

Fig. 4. Proposed domain structure of hormone-sensitive lipase.

nd 7 aspartate residues within the catalytic domain of rat HSL will provide an appropriate starting point for such a study.

Although the phosphorylation sites on HSL have been identified, little is known about the way changes in the phosphorylation state of the enzyme protein are expressed as changes in enzyme activity. This prompted an investigation of the effect of phosphorylation of the native enzyme at both phosphorylation sites on the protein structure in order to shed some light on the mechanism of activation and on the potentially antilipolytic effect of phosphorylation at the basal site [21]. Following phosphorylation by cyclic AMP-dependent protein kinase, radiolabelled HSL was subjected to limited tryptic digestion with increasing trypsin concentrations. Major [^{32}P]phosphoproteins were seen at 84 kDa (intact HSL) and a doublet at approx. 13 and 11.5 kDa which, on further digestion, resolved into the lower of the two bands. It appears that the phosphorylated regulatory site is contained within a relatively stable domain of 11.5 kDa (Fig. 3) which may constitute a regulatory domain within the HSL enzyme protein. Attempts to exactly localise this domain within the enzyme protein by N-terminal amino acid sequencing of the fragment, in a similar way to that described for the putative catalytic domain, were unsuccessful, possibly due to some artefactual blocking of the fragment.

However, when native HSL phosphorylated at the regulatory site is digested by trypsin both the catalytic domain containing the active site residue (as detected by labelling with ^{14}C]orlistat) and the domain containing the phosphorylation sites are recovered (compare Fig. 2, lane 3 and Fig. 3, lane 7), indicating that there is no overlap in sequence between these domains. Hence, the N-terminus of the phosphorylated domain must fall after the C-terminus of the catalytic domain approximately residue 499) but prior to the regulatory phosphorylation site (Ser-563).

In contrast to limited tryptic digestion of HSL phosphorylated at the regulatory site, no stable phosphorylated domain was produced following digestion of HSL phosphorylated at the basal site by AMP-activated protein kinase, nearly all the radioactivity being recovered in small phosphopeptides migrating at the dye front (not shown). One possible explanation for this surprising observation is that phosphorylation of the basal site produced a significant conformational change, rendering the phosphorylation domain more susceptible to proteolysis. Certainly the effects of phosphorylation at the two sites are very different in that phosphorylation of the regulatory site activates the enzyme whilst phosphorylation at the basal site has no apparent effect on activity but blocks subsequent phosphorylation at the regulatory site [21].

The ability of the putative catalytic domain described above to hydrolyse PNPB, but not lipid substrates, indicates that a further region of HSL may be necessary for lipid binding. It has been suggested previously that such a region lies close to the C-terminus of the polypeptide [3,4]. Consistent with this, a stable polypeptide of approximate mass 11.0 kDa was detected following digestion of HSL with low levels of trypsin which cause loss of activity against lipid substrates. Amino acid sequencing of this fragment indicated that the first 11 residues correspond to residues 658–668 in rat HSL (not shown). Based on 768 amino acids for intact rat HSL, the mass of approx. 11 kDa is consistent with this domain comprising the residues from 658 to the carboxyl-terminus of the HSL polypeptide and containing the hydrophobic sequence (FLSLAALC) (residues 735–742 of rat HSL), proposed to represent a possible lipid-binding site [4]. Recently, analysis of the mouse gene for HSL has shown the presence of an exon (exon 9) which may encode this putative lipid-binding domain [22].

Taken together with previous work, the data presented have allowed a possible domain structure for HSL to be proposed (Fig. 4), which is amenable to further test and experimentation. Of particular interest will be elucidation of a role for the 35 kDa in the amino-terminal region, since the three key properties of HSL, namely catalytic activity, phosphorylation sites and lipid binding capacity, can be ascribed to the carboxyl terminal region. One possible role for the N-terminal region would be in interacting with other regulatory components of the lipolytic and cholesterol metabolism pathways including possibly perilipin, a phosphoprotein implicated in HSL regulation [23].

Acknowledgements: This work was supported in part by a grant from the Biotechnology and Biological Sciences Research Council (UK), who also provided a research studentships to G.M.S. We thank Alan Harris for automated sequencer analysis.

References

[1] Yeaman, S.J. (1990) Biochim. Biophys. Acta 1052, 128–132.
[2] Rizack, M.A. (1961) J. Biol. Chem. 236, 657–662.
[3] Holm, C., Kirchgessner, T.G., Svenson, K.L., Fredrikson, G.,

Nilsson, S., Miller, C.G., Shively, J.E., Heinzmann, C., Sparkes, R.S., Mohandas, T., Lusis, A.J., Belfrage, P. and Schotz, M.C. (1988) Science 241, 1503–1506.
[4] Langin, D., Laurell, H., Holst, L.S., Belfrage, P. and Holm, C. (1993) Proc. Natl. Acad. Sci. USA 90, 4897–4901.
[5] Hemilä, H., Koivula, T.T. and Palva, I. (1994) Biochim. Biophys. Acta 1210, 249–253.
[6] Langin, D. and Holm, C. (1993) Trends Biochem. Sci. 18, 466–467.
[7] Garton, A.J., Campbell, D.G., Cohen, P. and Yeaman, S.J. (1988) FEBS Lett. 229, 67–72.
[8] Garton, A.J. and Yeaman, S.J. (1990) Eur. J. Biochem. 191, 245–250.
[9] Holm, C., Davis, R.C., Osterlund, T., Schotz, M.C. and Fredrikson, G. (1994) FEBS Lett. 344, 234–238.
[10] Tsujita, T., Ninomiya, H. and Okuda, H. (1989) J. Lipid Res. 30, 997–1004.
[11] Cordle, S.R., Colbran, R.J. and Yeaman, S.J. (1986) Biochim. Biophys. Acta 887, 51–57.
[12] Khoo, J.C., Steinberg, D., Huang, J.J. and Vagelos, P.R. (1976) J. Biol. Chem. 251, 2882–2890.
[13] Tornqvist, H., Bjorgell, P., Krabisch, L. and Belfrage, P. (1978) J. Lipid Res. 19, 654–656.
[14] Tsujita, T. and Brockman, H.L. (1987) Biochemistry 26, 8423–8429.
[15] Fredrikson, G., Strålfors, P., Nilsson, N.Ö. and Belfrage, P. (1981) J. Biol. Chem. 256, 6311–6320.
[16] Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599.
[17] Aitken, A., Geisow, M.J., Findlay, J.B.C., Holmes, C. and Yarwood, A. (1989) in: Protein Sequencing: A Practical Approach (Findlay, J.B.C. and Geisow, M.J. eds.) pp. 43–68, IRL Press, Oxford.
[18] Hochuli, E., Kupfer, E., Maurer, R., Meister, W., Mercadal, Y. and Schmidt, K. (1987) J. Antibiot. 40, 1086–1091.
[19] Borgstrom, B. (1988) Biochim. Biophys. Acta 962, 308–316.
[20] Hadvary, P., Sidler, W., Meister, W., Vetter, W. and Wolfer, H. (1991) J. Biol. Chem. 266, 2021–2027.
[21] Garton, A.J., Campbell, D.G., Carling, D., Hardie, D.G., Colbran, R.J. and Yeaman, S.J. (1989) Eur. J. Biochem. 179, 249–254.
[22] Li, Z., Sumida, M., Birchbauer, A., Schotz, M.C. and Reue, K. (1994) Genomics 24, 259–265.
[23] Londos, C., Gruia-Gray, J., Brasaemie, D.L., Rondinone, C.M., Takeda, T., Dwyer, N.K., Barber, T., Kimmel, A.R. and Blanchette-Mackie, E.J. (1996) Int. J. Obesity 205, 97–101.