Domain Identification of Hormone-sensitive Lipase by Circular Dichroism and Fluorescence Spectroscopy, Limited Proteolysis, and Mass Spectrometry*

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Structure-function relationship analyses of hormone-sensitive lipase (HSL) have suggested that this metabolically important enzyme consists of several functional and at least two structural domains (Østerlund, T., Daniellson, B., Deitmerman, E., Contreras, J. A., Edgren, G., Davis, R. C., Schotz, M. C., and Holm, C. (1996) Biochem. J. 319, 411–420; Contreras, J. A., Karlsson, M., Østerlund, T., Laurell, H., Svensson, A., and Holm, C. (1996) J. Biol. Chem. 271, 31426–31430). To analyze the structural domain composition of HSL in more detail, we applied biophysical methods. Denaturation of HSL was followed by circular dichroism measurements and fluorescence spectroscopy, revealing that the unfolding of HSL is a two-step event. Using limited proteolysis in combination with mass spectrometry, several proteolytic fragments of HSL were identified, including one corresponding exactly to the proposed N-terminal domain. Major cleavage sites were found in the predicted hinge region between the two domains and in the regulatory module of the C-terminal, catalytic domain. Analyses of a hinge region cleavage mutant and calculations of the hydrophatic pattern of HSL further suggest that the hinge region and regulatory module are exposed parts of HSL. Together, these data support our previous hypothesis that HSL consists of two major structural domains, encoded by exons 1–4 and 5–9, respectively, of which the latter contains an exposed regulatory module outside the catalytic α/β-hydrolase fold core.

The release of fatty acids from stored triglycerides in adipocytes is accomplished by hormone-sensitive lipase (HSL) and monoglyceride lipase. Lipolysis is regulated by hormones and neurotransmitters, and the major target of this regulation is HSL (1, 2). Catabolic hormones and neurotransmitters (e.g. norepinephrine) activate cAMP-dependent protein kinase, which, in turn, phosphorylates and activates HSL. The major antilipolytic hormone is insulin, which activates phosphodies-

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1 The abbreviations used are: HSL, hormone-sensitive lipase; DTE, dithioerythritol; GdnCl, guanidine hydrochloride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

2 The residue numbering is for rat HSL.
11). This part is a substrate of kinases and phosphatases and has been predicted to contain only a few secondary structure elements (11). Thus, it is suspected to be an exposed and rather flexible module. The N-terminal part of the protein (approximately 320 residues) is believed to constitute a separate structural domain and shows no significant sequence similarity to other proteins (10).

To establish the structural domain composition of HSL, we have performed spectroscopic analyses during denaturation. Data from these analyses support the idea that HSL has two major structural domains. Limited proteolysis analyses (10) have been extended through identification of generated peptides by mass spectrometry to identify exposed regions of the intact protein and to identify more stable fragments of the HSL molecule. Both the suggested hinge region and the regulatory module are exposed to proteolysis, whereas the N-terminal domain appears to be highly resistant to cleavage. Analysis by proteolysis of a HSL mutant with an engineered Factor X site in the predicted hinge confirms these results, and further support is provided by analysis of the hydrophatic pattern of HSL. Based on the generated results, we present a more complete overall structural model for HSL.

EXPERIMENTAL PROCEDURES

Recombinant HSL—Recombinant rat HSL was expressed and purified as described previously (10, 14). After this purification, which includes the use of Q- and phenyl-Sepharose columns, HSL was concentrated using small (1–3 ml) Q-Sepharose columns (14). HSL was applied to these columns either directly in the phenyl-Sepharose elution buffer or after dialysis against 20 mM Tris acetate, pH 7.5, 1 mM dithioerythritol (DTE), 0.2% C12E12 (a non-ionic alkyl polyoxyethylene ether-type detergent; see Ref. 14), and 10% glycerol. Washing was performed with Buffer A (50 mM Tris acetate, pH 7.5, and 1 mM DTE) supplemented with different types and concentrations of detergents and different concentrations of glycerol, depending on the type of analysis after concentration (14). Elution was performed in one step with the particular Buffer A supplemented with 300 mM sodium acetate.

Circular Dichroism Spectroscopy—HSL subjected to denaturation by guanidine hydrochloride (GdnCl) or thermal denaturation was monitored using circular dichroism spectroscopy (CD) on a J-720 spectropolarimeter (Jasco). HSL samples (50 μl; 2 mg/ml in Buffer A with 0.2% C12E12 and 5% glycerol) were mixed with 200 μl of Buffer P (100 mM potassium phosphate, pH 7.25, 0.9% sodium chloride, and 1 mM DTE) with different concentrations of GdnCl, and the CD signal was measured at 222 nm in a 1-mm cuvette. Final concentrations of glycerol and detergent (C12E12) were 1% and 0.04%, respectively. After measurements by CD, the samples were recovered and stored at −80 °C until they were analyzed for fluorescence spectroscopy (see below).

For analyses during thermal denaturation, 200 μl of HSL (0.4 mg/ml) in 5 mM potassium phosphate, pH 7.4, 1 mM DTE, 50% glycerol, and 0.2% C12E12 were mixed with 300 μl of Buffer P without GdnCl and monitored by CD at a gradually increasing temperature ranging from 4 °C to 90 °C.

Fluorescence Spectroscopy—Fluorescence measurements were performed on a LS50B luminescence spectrometer (Perkin-Elmer). HSL samples (200 μl) recovered from the CD measurements were further diluted with either 600 μl of Buffer P or Buffer P supplemented with 0.04% C12E12 and 1% glycerol, containing the same concentration of GdnCl as the original sample. All samples were then excited at both 280 and 295 nm, and data were collected from 305 to 420 nm. 50 μl of glycerol or 10 μl of 20% C12E12 were then added to the samples diluted in Buffer P, and 50 μl of glycerol were added to the samples diluted in detergent/glycerol-supplemented buffer P, and then the fluorescence was measured again.

Analytical Ultracentrifugation—For sedimentation equilibrium centrifugations of HSL, Brij-96 was used as detergent instead of C12E12 because the partial specific volume of Brij-96 is close to 1. The exchange of C12E12 for Brij-96 was performed on the last Q-Sepharose column used in the purification of recombinant HSL (see above). After application of the enzyme from the phenyl-Sepharose step, the column was washed with 50 column volumes of 50 mM Tris acetate, pH 7.5, 0.1 mM DTE, 5% glycerol, and 0.006% Brij-96 (Fluka). Elution was performed in the same buffer supplemented with 0.3 mM sodium acetate, and then the pooled material was dialyzed against phosphate-buffered saline, 0.1 mM DTE, 5% glycerol, 0.006% Brij-96, and 0.5 mM EDTA. Sedimentation equilibrium centrifugations were performed at an initial HSL concentration of 0.08 mg/ml in the absence and presence of 0.2 M GdnCl in a Beckman Optima XL-A analytical centrifuge at 7,000 rpm at 4 °C, using absorption optics and data analysis software provided by the manufacturer.

Proteolysis—The HSL preparation (2 mg/ml) used for CD and fluorescence analysis was also used for proteolytic digests. Endoprotease Lys-C (EndoL) was changed to 3 units/ml in the supplied buffer (Promega). Both initial and comprehensive proteolysis of HSL were performed in the same reaction. Proteolysis was initiated by mixing 50 μl of HSL with 1 μl of protease at room temperature. Aliquots of 5 μl were taken for analysis by SDS-PAGE at three time points up to 5 min. At 5 min, an additional 5 μl of protease were added, and proteolysis continued for an additional 90 min. From the more extensive digest, five more aliquots of 6 μl were taken for SDS-PAGE analysis. All aliquots were added directly to tubes containing 2× SDS-PAGE loading buffer.

SDS-PAGE and Peptide Blotting—The HSL digests were analyzed on SDS-PAGE using the Tris/HCl-tricine buffer system (15). Samples from initial digests were run on 8% gels, and samples from the more extensive digests were run on 12% gels. After electrophoresis, peptides were blotted onto polyvinylidene difluoride membranes (Immobilon P; Millipore) and visualized with 0.1% Ponceau Red (Sigma).

Mass Spectrometric Analysis—From the HSL peptide blots, distinct bands were excised, destained, and digested in 200 μl sodium hydroxide solution and dialyzed in water. The membrane pieces were then subjected to digestion using sequencing-grade modified trypsin (Promega) at 37 °C overnight. The tryptic peptides were separated on a C18 reverse phase high pressure liquid chromatography microcapillary column using a gradient from 0% to 30% acetonitrile in 0.1% trifluoroacetic acid and eluted into a trap quadrupole mass spectrometer (Finnigan) equipped with an electrospray ionization source. Several tryptic peptides from each EndoL-derived HSL fragment were subjected to collision-induced dissociation analysis, generating even smaller heterogenous and overlapping peptide fragments from which the primary structures were deduced.

HSL Cleavage Mutant—A cleavage mutant of rat HSL was constructed by a polymerase chain reaction-based overlap extension (12). The details are described in the legend to Fig. 2. To establish the structural domain composition of HSL, we have generated even smaller, heterogeneous and overlapping peptide fragments from which the primary structures were deduced.

HSL Cleavage Mutant—A cleavage mutant of rat HSL was constructed by a polymerase chain reaction-based overlap extension. All mutants were generated using the BaculoGold transfection kit as described by the manufacturer (PharMingen). Production in Sf9 cells and subsequent purification of the mutant HSL (HSLFacX), was performed as described for the wild-type HSL (see above). The purified mutant was subjected to proteolysis at 37 °C with Factor Xa (New England Biolabs) by mixing 10 μl of HSLFacX (2 mg/ml in Buffer A with 0.2% C12E12, 10% glycerol, and 300 mM sodium acetate) with 39 μl of 20 mM Tris/HCl, pH 7.5, 100 mM sodium chloride, 2 mM calcium chloride, and 1 μl of protease (1 mg/ml). The digestion mix was incubated at different time points and subjected to SDS-PAGE analysis on 8% gels as described above. Peptides were visualized by staining with Coomassie Brilliant Blue (Serva). A control, the same procedure was performed with wild-type HSL.

RESULTS

Denaturation Experiments—To analyze the unfolding of HSL, CD and fluorescence spectroscopy were used to follow changes in the secondary and tertiary structure, respectively, brought about by increasing GdnCl concentrations. For the fluorescence spectroscopy, two different series of denaturation were created. One series had identical detergent and glycerol concentrations as the CD measurements, and one had lower detergent and glycerol concentrations (diluted in salt buffer). Fig. 1 shows the fluorescence scans of HSL in low GdnCl (20 mM) and high GdnCl (5.44 M) concentrations, thus comparing the folded and denatured states. The spectrum appears to be composed of three overlapping peaks, which two decrease significantly upon denaturation (peak 1 at 321 nm and peak 2 at 333 nm). At these wavelengths, only tryptophan residues have fluorescence emission. Five tryptophans are found in rat HSL, two are found in the suggested N-terminal domain (Trp-238 and Trp-242), two are found in the α/β-hydrolase fold core (Trp-368 and Trp-402), and one is found in the regulatory domain.
domain (Trp-525). An isofluorescent point was identified at 362 nm. The fluorescence at peak 1 and peak 2 was normalized for all samples using the value at 362 nm to compensate for the differences in HSL concentrations between samples. The results of these calculations are shown in Fig. 2. Only results from excitation at 280 nm measured at peak 1 are shown, because very similar results were obtained at peak 2 and when samples were excited at 295 nm. It is clear that loss of fluorescence takes place in two steps, at a C_m of approximately 0.15 and 3.5 mM GdnCl, respectively, indicating the unfolding of two independent domains. Gel filtration studies using small amounts of rat HSL purified from adipose tissue have suggested that HSL may exist as a homodimer in solution (1). The loss in fluorescence at a low GdnCl concentration may therefore arise due to the dissociation of dimers. We have initiated analyses of the subunit composition of HSL by ultracentrifugation and gel filtration. The results obtained strongly indicate that HSL is a homodimer (Fig. 3). Ultracentrifugation experiments of HSL in the presence of GdnCl were also performed. The results of sedimentation equilibrium of HSL at an initial concentration of 0.08 mg/ml are presented in Fig. 3. The equilibrium gradients, both in the absence and presence of 0.2 mM GdnCl, are best fit by a single species of about 170,000 Da, and the goodness of fit is indicated by the random distribution of the residuals. Therefore, at the concentration used for fluorescence measurements, there is no significant dissociation of dimers at 0.2 mM GdnCl. Thus, it is likely that most of the loss in fluorescence at low GdnCl concentrations is due to changes in the tertiary structure. It is clear from Fig. 2 that HSL is stabilized in the detergent and glycerol series as compared with the salt-diluted series. This is probably due to the interaction of these compounds with the protein, shielding it from GdnCl and shifting the denaturation toward higher GdnCl concentrations. The effects of detergent and glycerol are most markedly seen during denaturation of the more stable domain. Repplotting of the series diluted in salt buffer and the series diluted in detergent/glycerol-supplemented salt buffer in the range from 1 to 6 mM GdnCl (data not shown) allowed estimations of the changes in free energy of transition from the native (N) to the unfolded (U) state in the absence of denaturant (ΔG_N→U(H_2O)) and midpoint concentrations of GdnCl (C_m) for the unfolding (17) of this domain. The salt-diluted and detergent/glycerol/salt-diluted series had an estimated ΔG_N→U(H_2O) of 17 and 21 kJ/mol and a C_m of 3.1 and 3.8 mM GdnCl, respectively. This confirms that there is a marked effect of detergent and glycerol on the unfolding of this particular domain.

Additions of glycerol to either series had only marginal effects (data not shown), whereas the addition of detergent (0.23% final concentration) to the salt-diluted series markedly increased relative fluorescence in the range from 1 to 5 mM GdnCl. Whether this addition induces some refolding or the detergent interacts directly with tryptophans to increase fluorescence is not clear. Because glycerol on its own had very little effect, it is likely that the stabilizing effect in the detergent/glycerol/salt buffer-diluted series is mainly due to the detergent.

Fig. 4 shows the normalized CD measurements of HSL with increasing GdnCl. The unfolding seems quite complex, but two major reductions in the secondary structure content can be seen at 0.5–1.5 and 2.5–4 mM GdnCl, respectively. The fluorescence data from the detergent/glycerol-diluted series have been replotted (Fig. 4) because these are measured under the same conditions of detergent and glycerol as the CD measurements. The reduction in fluorescence does not seem to be accompanied by a loss of secondary structure content from 0 to 0.4 mM GdnCl. In contrast, as GdnCl concentration increases to 1.5 mM, there is a substantial loss in the CD signal but only a small reduction in fluorescence. From 2.5 to 4 mM GdnCl, there is a clear reduction in both the secondary and tertiary structure, corresponding to the complete unfolding of a second domain. It is possible to envisage that at low GdnCl concentrations, one domain unfolds without the disruption of secondary structure elements (particularly α-helices). Then, as GdnCl increases from 0.5 to 1.5 mM, the secondary structure elements of this domain unfold with a C_m of approximately 0.9 mM GdnCl and only minor additional exposure of fluorescent side chains. Above 2.5 mM GdnCl, both the exposure of fluorescent side chains and the disruption of secondary structure elements in a second domain occur. Of course, the general picture can be reconciled with the presence of even more structural domains in HSL, but hardly with only one domain. It is noteworthy that the loss in fluorescence below 0.5 mM GdnCl coincides with the loss of HSL activities measured at these concentrations of GdnCl (10). This suggests that it is primarily the C-terminal domain, harboring the catalytic core/machinery (11), that is affected at these low GdnCl concentrations.

HSL was also analyzed by CD during thermal denaturation.

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3 P. Poon, T. Østerlund, M. C. Schotz, C. Holm, and H. Wong, unpublished results.
Fig. 5 shows the result of one such experiment, in which it can be seen that the reduction in the secondary structure content takes place in two steps. Absorption measurements (data not shown) indicate that some protein aggregation takes place during thermal denaturation. However, comparing CD scans from different temperatures (data not shown) and comparing the results in Fig. 5 with the results of a thermal denaturation in which complete aggregation occurred (data not shown) provide evidence that protein aggregation does not contribute to the loss in the CD signal. Together, the spectroscopy data strongly support the notion that HSL is composed of two major structural domains.

Proteolytic Analysis—To identify those putative proteolytic sites in HSL that are accessible to enzymatic cleavage, we turned to limited proteolysis. From our previous study using limited proteolysis, it was clear that lipase activity (hydrolysis of phospholipid-stabilized lipid substrate emulsions) demanded an almost intact protein, whereas esterase activity (hydrolysis of soluble substrates) could be measured after extensive proteolysis (10). Among the regions in the suggested domain structure (10, 11) predicted to be particularly accessible to proteolysis are the regulatory module (approximately residues 460–680), because this probably contains few secondary structure elements (11) and serves as a substrate for kinases and phosphatases, and the putative hinge region between the two structural domains (approximately residue 315–335). HSL was digested with EndoL, and the peptides generated were identified by mass spectrometry. Both initial proteolysis with a low concentration of EndoL and extensive digestion with a much higher concentration of protease were performed. Fig. 6 shows the generated HSL peptides on a nylon membrane. Several products are observed in the initial digest (Fig. 6A), of which one is more abundant, whereas three stable fragments can be seen even after 90 min of extensive proteolysis (Fig. 6B). The major band appearing after initial proteolysis (p64) corresponds to residues 1–583. Therefore, a major site of cleavage is probably located at Lys-583 in the anticipated regulatory module. The three more stable bands (p36, p25, and p20) cover three different parts of HSL. The most abundant is p36, which encompasses residues 1–323 and thus corresponds exactly to the suggested N-terminal domain (10). Another major band is p25, which corresponds to the C-terminal 229 amino acids (540–768). Because cleavage at Lys-539 (generation of p64) and Lys-583 (generation of p44) cannot generate both p25 and p64, there must be more than one pathway of cleavage. In the

Fig. 3. Sedimentation equilibrium of HSL in the absence and presence of GdnCl. HSL at an initial concentration of 0.08 mg/ml was analyzed by sedimentation equilibrium centrifugation as described under “Experimental Procedures” in the absence (A) and presence (B) of 0.2 M GdnCl. Nonlinear square curve fitting of the equilibrium gradients, absorbance (at 232 nm in A and at 234 nm in B) versus radius, gave molecular weights of 164,000 for A and 176,000 for B. The residuals (the differences between the data and curve fit) are random, indicating that a single species is a reasonable fit.

Fig. 4. GdnCl denaturation of HSL measured by CD and fluorescence spectroscopy. Changes in relative CD signal at 222 nm (▼) and fluorescence at peak 1 (○) were measured as a function of GdnCl concentration under the same conditions with respect to detergent and glycerol. The fluorescence data are replotted from Fig. 2. The CD data points are from one experiment repeated three times.

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The thermal denaturation of HSL was measured by CD. Denaturation of HSL by increasing temperature was measured at 222 nm and expressed as a normalized CD signal. Single data points are from one experiment repeated four times.

**FIG. 6.** Proteolytic digestion of HSL by EndoL. HSL was subjected to limited proteolysis by EndoL at (A) low and (B) high concentrations of protease. HSL peptides were analyzed by SDS-PAGE on 8% (A) and 12% (B) gels, respectively. After electrophoresis, peptides were identified by staining with Ponceau Red. Lanes 2–4 are proteolytic digests for 0.5, 2, and 5 min, respectively. Lanes 6–10 are digests for 2, 10, 30, 60, and 90 min, respectively, after a further addition of EndoL (see “Experimental Procedures”). The different peptides that have been identified are indicated to the right of B, and the residues that they represent in HSL, based on mass spectrometric analyses, are indicated in parentheses. Tentative assignments are indicated by question marks.

The generation of p25, cleavage has taken place at another site in the regulatory module as compared with the cleavage that generates p64. In fact, in these two pathways, cleavage takes place at opposite sides of two of the phosphorylation sites (Ser-563 and Ser-565). The nature of the p20 band has not been definitely established because it appears that at least two peptides are present. However, there is strong evidence that one peptide is residues 359–540. This fits well with a previously identified stable tryptic peptide in bovine HSL corresponding to residues 333–499 (18). The p20 (residues 359–540) peptide, together with p36 and p25, covers almost the entire HSL protein. There is also evidence that another peptide in the p20 band could be residues 1–161. This would indicate that p36 is not completely resistant to proteolysis, although it takes several hours of continued proteolysis to observe a reduction in the intensity of this band (data not shown).

**HSL Cleavage Mutant**—Encouraged by the finding that proteolysis takes place in the predicted regions according to the domain structure model (see above), we constructed a mutant of HSL with a specific cleavage site for Factor X (HSLFacX) in the proposed hinge region. The mutant HSL was expressed in insect cells to the same level as the wild-type protein (data not shown). After purification of HSLFacX, its specific activity was estimated to be the same as that of wild-type HSL (10). Cleavage by Factor X takes place at an introduced Arg-323 in the putative hinge region between the two suggested structural domains. This arginine replaces Lys-323 that was shown to be the accessibility to the protease. After cleavage of the two domains, the C-terminal domain is subjected to further nonspecific cleavage, whereas the N-terminal domain is not. Under the same conditions, there is also significant nonspecific cleavage of the wild-type protein. From the pattern of cleavage, it is suggested that there are three major nonspecific cleavage sites for Factor X in wild-type HSL, probably all in the regulatory module. This suggestion is based on two observations. First, the three larger bands range from 50 to 70 kDa in size, comparable to the major band generated initially by EndoL (Fig. 6), and secondly, only the C-terminal domain of HSLFacX undergoes any further significant cleavage. Intriguingly, some of the smaller bands generated by cleavage of this domain correspond exactly in size to bands in the wild-type HSL digest.

Attempts to crystallize HSL have been unsuccessful thus far, most likely due to the demand for detergent in HSL preparations, heterogeneity of the protein (it has four phosphorylation sites), and instability of the protein (see above). To generate the separate domains for further crystallization attempts, procedures for the purification of the two domains after cleavage of HSLFacX will be worked out.

**DISCUSSION**

In this study, further evidence for our previously proposed model for the domain structure of HSL is provided (10, 11). The denaturation analyses support that HSL has at least two (and probably only two) structural domains. The fluorescence data clearly suggest the unfolding of two individually folded domains. Although HSL appears to be a homodimer in solution, it is unlikely that the dissociation of dimers contributes significantly to the loss in fluorescence at low GdnCl concentrations. CD measurements during denaturation by GdnCl and heat indicate that the unfolding of secondary structure elements occurs in two major steps. It cannot be ruled out that some protein aggregation occurs in the thermal denaturation. However, it does not seem to have any significant influence on the
The mean hydropathy index was calculated in sections of 60 amino acids using the Kyte and Doolittle (19) algorithm in the GeneWorks program (IntelliGenetics Inc.) with a span of 11 residues, as recommended. The mean hydropathy indices are directly compared with the proposed domain structure as outlined in the schematic primary structure (bar). The N-terminal domain is dark gray, the hinge region is black, the regulatory part is light gray, and the α/β-hydrolase fold core is white. Arrows indicate the major proteolytic cleavage sites determined in Fig. 6.

From both spectroscopic data and proteolytic analyses, it appears that one domain is more fragile than the other. The fragile domain is unfolded by low GdnCl concentrations and probably represents the C-terminal domain, because all HSL activities are lost concomitant with its unfolding (10). This domain is also highly sensitive to proteolytic digestion, especially in the regulatory module, as shown by proteolytic cleavage by both EndoL and Factor X. The second unfolding, which occurs at high GdnCl concentrations, probably represents the N-terminal domain. Because detergent and perhaps glycerol had a more protective effect on this domain than on the other domain, it is speculated that some sites of detergent and membrane interactions are located here. Other sites might be found in the α/β-hydrolase fold core, although most hydrophobic residues of this part are not exposed (11). To directly compare the pattern of hydrophobicity in the primary structure of HSL with the proposed domain structure, the mean hydropathic index was calculated for every 60 residues, according to Kyte and Doolittle (19). Fig. 8 shows the calculated hydropathy indices with indications of the localization of suggested domains in the primary structure. Hydrophilic regions correlate well with the anticipated hinge region and regulatory module. Arrows indicate the major cleavage sites as identified in Fig. 6. The most hydrophobic regions are those of the α/β-hydrolase fold, whereas the N-terminal domain has an amphipathic character. Because the regulatory module is probably located at the surface of the C-terminal domain, and most of the hydrophobic residues of the α/β-hydrolase fold are located in the core of the fold (11), there are probably only a few exposed hydrophobic patches in this part. The membrane/detergent binding sites are presumably located at these patches as well as at hydrophobic patches in the N-terminal domain. Overall, the hydropathy calculations support the suggested domain structure, particularly by demonstrating that the exposed regions are markedly hydrophilic.

The exact location of the hinge region in the primary structure has been the subject of some speculation (10, 11). The cleavage at Lys-323 and the hydrophilic character of this region support the concept that the N- and C-terminal domains are separated at the hinge region (residues 315–335) as suggested from alignments (10). Cleavage of HSL parentheses by Factor X shows that this region is immediately accessible to the protease. Our current view and working hypothesis of the domain structure is illustrated in Fig. 9 with indications of cleavage sites and action of GdnCl at different concentrations. The secondary structure elements of the α/β-hydrolase fold are outlined (11).

In conclusion, the present study provides additional support for the proposal that HSL is composed of two major structural domains encoded by exons 1–4 and 5–9, respectively. The latter is the catalytic domain formed by an α/β-hydrolase fold core (11), which is interrupted in the primary structure by the insertion of a regulatory module. Both the regulatory module and the suggested hinge are exposed hydrophilic parts. Additional investigations of HSL parentheses before and after cleavage and the expression of individual domains are underway. Purified domains and fragments will be valuable tools for structural and functional analyses as well as the determination of the three-dimensional structure of HSL.

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