bis-ANS acts as an inhibitor of lipoprotein lipase and competes for binding with apolipoprotein CII*

by

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Summary

Lipoprotein lipase (LPL) is dependent on apolipoprotein CII (apoCII), a component of plasma lipoproteins, for function in vivo. The hydrophobic fluorescent probe 1,1'-bis(anilino)-4-,4'-bis(naphtalene)-8,8'-disulfonate [bis(ANS)] was found to be a potent inhibitor of LPL. ApoCII prevented the inhibition by bis-ANS, and was also able to restore the activity of inhibited LPL in a competitive manner, but only with triacylglycerols with acyl chains longer than three carbons. Studies of fluorescence and surface plasmon resonance indicated that LPL has an exposed hydrophobic site for binding of bis-ANS. The high affinity interaction was characterized by an equilibrium constant $K_d$ of 0.10 to 0.26 µM, and by a relatively high on rate constant $k_{on}= 2.0 \times 10^4 \ M^{-1}s^{-1}$ and a slow off rate with a dissociation rate constant $k_{diss} = 1.2 \times 10^{-4} \ s^{-1}$. The high affinity binding of bis-ANS did not influence interaction of LPL with heparin or with lipid/water interfaces and did not dissociate the active LPL dimer into monomers. Analysis of fragments of LPL after photoincorporation of bis-ANS indicated that the high-affinity binding site was located in the middle part of the amino-terminal folding domain. We propose that bis-ANS binds to an exposed hydrophobic area that is located close to the active site. This area may be the binding-site for individual substrate molecules and also for apoCII.
Introduction

Lipoprotein lipase (LPL) is one of the central proteins in blood lipid metabolism (for recent reviews see 1-3). The enzyme is bound to heparan sulfate proteoglycans at the vascular endothelium and hydrolyses triacylglycerols and phospholipids in plasma lipoproteins so that lipolysis products can be taken up in adjacent cells for metabolic purposes. In addition, and independent of catalysis, the LPL protein functions as a mediator for binding and uptake of lipoproteins by cells by bridging between the lipoproteins and heparan sulphate proteoglycans or receptors of the LDL-R family.

LPL belongs to the family of mammalian triglyceride lipases together with pancreatic lipase, hepatic lipase and endothelial lipase (2). Based on sequence homology with pancreatic lipase, models of the 55 kD subunit of LPL have been created (4-6). The active form of LPL is a non-covalent dimer of identical subunits (7;8), which are arranged in a head-to-tail fashion (5;9). LPL can engage in a number of interactions, with heparin/heparan sulphate, receptors, lipid/water interfaces, the activator apolipoprotein CII (apoCII) and perhaps with other apolipoproteins. Fatty acids with long acyl chains have been shown to bind to LPL with high affinity (10) and to inhibit the catalytic activity of LPL as well as binding of LPL to lipid/water interfaces (lipoproteins, emulsion droplets) (11) and to heparin (12). Thus, fatty acids exert strong control over the activity of LPL. Their removal by uptake in tissues, or by binding to albumin, is essential to allow the action of the enzyme to proceed unabated.

ApoCII, a 79 amino acid residue peptide belonging to the apoC family of exchangeable apolipoproteins, is a necessary activator for LPL in vivo (13;14). Deficiency of apoCII leads to massive accumulation of lipids in blood and symptoms similar to what is seen on LPL deficiency (15). Systematic mutagenesis of residues in the LPL binding domain of apoCII and studies of the three-dimensional structure of apoCII by NMR, strongly suggest that the basis for the activation is formation of a complex between apoCII and LPL at the surface of a lipid droplet (16;17). While the region of apoCII responsible for the interaction with LPL has been localized to the C-terminal part of the peptide (18), it is still unclear which part(s) of LPL that interact with apoCII. From studies of chimeras of LPL with the related hepatic lipase it has been concluded that apoCII may interact across the anti-parallel LPL dimer with binding-sites both on the N-terminal and C-terminal folding domains of LPL (19). By computer modelling of the molecular docking of a C-terminal fragment of apoCII, spanning residues 50-79, to the LPL monomer, the binding site(s) for apoCII was suggested to be located at the interface between the N-terminal and C-terminal folding domains of LPL.
and also engaging the lid region that covers the active site (6). A different interaction site was recently reported by McIlhargey et al based on experiments with cross-linking and mutagenesis (20). They proposed that LPL residues 65-68 and 73-79 are involved in the activation by apoCII. In the model of LPL these residues are part of a helix located in close proximity to the active site pocket.

The molecular mechanism for the activation of LPL by apoCII is still unresolved. Both proteins bind independently to lipid droplets (21;22). Thus, apoCII is not needed for interfacial binding of LPL. ApoCII has effects on LPL activity only with triacylglycerols and phospholipids that contain acyl chains longer than 7 carbons (23-25). The level to which apoCII activates LPL in vitro depends not only on the substrate molecules, but also on components like surfactants and other proteins that are present in the system (21). The conditions when apoCII has effects on LPL activity, i.e. in the presence of emulsified lipids, are not easily compatible with biophysical techniques for studies of protein structure and interactions. Therefore, most information comes from indirect studies of enzyme kinetics and from studies of mutants of apoCII and LPL.

In the present study we have used the aromatic hydrophobic probes 1,1'-bis(anilino)-4-,4'-bis(naphthalene)-8,8'-disulfonate [bis(ANS)], 1-anilinonaphthalene-8-sulfonic acid (ANS) and triiodotyronin (T3) to investigate the mechanism of LPL action. Bis-ANS and ANS are often used as sensitive probes to detect hydrophobic sites and conformational changes in proteins (26-28). An additional advantage with these compounds is the possibility to study irreversible inhibition after photoincorporation by irradiation with UV light (29). We show that bis-ANS and ANS can be used to obtain specific information about the interaction of LPL with substrates and apoCII. Our results suggest that bis-ANS binds to an exposed hydrophobic site at or close to the binding site(s) for apoCII and for individual substrate molecules. A similar site is not present in pancreatic lipase or bile salt-stimulated lipase. We propose that apoCII binds close to the substrate binding site on LPL and that the activator may play an important role for the alignment of individual substrate molecules into the active site.

Materials and methods

Materials - LPL was purified from bovine milk as described (30). Bis-ANS and ANS were from Molecular Probes. Mutants of apoCII were expressed and purified as described in a
previous study (16). The apoCII fragment spanning residues 50-79 was synthesized for a previous study (31). Pancreatic lipase and colipase were purified from porcine pancreas as previously described (32;33). Bile salt-stimulated lipase purified from human milk was a kind gift from Dr Lars Bläckberg, Umeå University. Tributyrin, tripropionin, triacetin, tricaprylin p-nitrophenylbutyrate, bovine serum albumin (fraction V) and gum arabic were obtained from Sigma. 125I –LPL was prepared by the lactoperoxidase method and purified on heparin-Sepharose (34). Inactive, monomeric LPL was prepared by treatment with 1M guanidinium chloride as previously described (8;34). The concentration of bis-ANS was determined by absorbance at 394 nm using A_{394} = 16000 cm^{-1}M^{-1} (35).

**Conditions for pre-treatment of lipases by bis-ANS** - Incubations with LPL were made under conditions when the enzyme was as stable as possible. Depending on the purpose for the experiment this was done either at 10° C in 20 mM Tris-Cl, 0.5 M NaCl, pH 7.4 or at 25° C in 20 mM Tris-Cl, pH 8.5, containing 4 mM sodium deoxycholate. Stock solutions of bis-ANS (5 µM to 10 mM) were made in methanol. Pancreatic lipase and bile stimulated lipase were incubated with bis-ANS at 25° C in 20 mM Tris-Cl, pH 7.4, in the presence of 0.5 M or 0.15 M NaCl, respectively.

**Enzyme assays** - Most of the activity measurements with triacylglycerols as substrates were performed by continuous titration of fatty acids released using a pH-stat (Methrome, Herisau) at 25° C. For these experiments stock solutions of the substrates were prepared by sonication of the triacylglycerols in gum arabic/water solution (MSE Soniprep). The enzymatic activity was recorded at pH 8.5 in 0.15 M NaCl containing 30 mM triacylglycerol and 0.2 % gum arabic. In several experiments the reaction medium also contained bovine serum albumin as specified in the figure legends.

The activity of LPL against p-nitrophenyl butyrate was measured at pH 7.4 at room temperature in 0.1 M phosphate buffer containing 15 IU heparin/ml and 0.15 M NaCl. The concentration of substrate ranged between 0.1-0.5 mM. The release of p-nitrophenol was continuously monitored at 400 nm.

**Sucrose density gradient centrifugation** - For determination of the aggregation state of LPL/bis-ANS complexes, sedimentation was performed at 10° C in linear gradients of sucrose (5-20 %). The gradients (3.6 ml) were made in 20 mM Tris, 1.5 M NaCl, 1 mg/ml BSA, pH 7.4. Samples of LPL in the same buffer (0.5 µM, 200 µl total volume) were preincubated
with bis-ANS in 2-fold and 20-fold molar excess, respectively, for 1 h and were then applied on top of the gradients, which also contained the corresponding concentrations of bis-ANS. Centrifugation was for 18 h in a Beckman Coulter SW 60 rotor and fractions of 0.24 ml were collected from the bottom of the tubes. In this case, activity measurements were made using $^3$H-triolein-labelled Intralipid (10 %) as substrate (kindly prepared by Pharmacia Upjohn, Stockholm Sweden), and the assay contained in addition to the long-chain triacylglycerols, 1 mM BSA and 5 % rat serum as a source of apoCII to promote regain of activity of the inhibited enzyme (16).

**Heparin-Sepharose chromatography** – This was carried out at 4 ºC on a small column (2 ml gel) equilibrated with 20 mM Tris-Cl, pH 7.4, 0.15 M NaCl and 10 % glycerol. After application of the sample, and wash with buffer without NaCl, LPL was eluted by a linear gradient of NaCl from 0 M to 1.5 M. The salt gradient was determined by conductometry using known concentrations of NaCl made up in the same buffer as a standard.

**Measurements of fluorescence and circular dichroism** - Fluorescence measurements were performed using a Spex FluoroMax-2 fluorometer. The experiments were done either at 25º C in 20 mM Tris-Cl, pH 8.5, containing 4 mM sodium deoxycholate or at 10º C in 20 mM Tris-Cl, pH 8.5, containing 15 IU heparin/ml and 0.15 M NaCl. Stock solutions of bis-ANS were prepared in methanol. LPL concentrations varied between 30-100 nM. Circular dichroism (CD) measurements were carried out using a JASCO J-700 spectropolarimeter. The experiments were performed at 10º C, in 20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl.

**Binding studies on BIAcore** - The binding studies were performed on a BIAcore 3000 instrument using sensorchips CM5. Most of the experimental details concerning immobilization of heparin and kinetic analyses of data are described in previous studies (36;37). The binding experiments were carried out at 25º C, in 10 mM hepes buffer, pH 7.4, containing 0.15 M NaCl. To estimate how many bis-ANS molecules that bound per LPL, we used the established relationship that for proteins a surface density of 1 ng/mm² increases the response by about 1000 units. We assumed that this relationship was valid also for bis-ANS (38).
**Photoincorporation of bis-ANS** – This was carried out according to Seale *et al.* (29). Briefly, samples of LPL in 20 mM Tris-Cl, 0.5 M NaCl, pH 7.4 or in 20 mM Tris-Cl, pH 8.5, 4 mM deoxycholate, in the presence of various concentrations of bis-ANS, were irradiated at 4°C by UV light (250 nm). For comparison, samples of LPL without bis-ANS, were given the same treatment. After irradiation, unbound bis-ANS was removed by extensive dialysis against 0.05% SDS, or against the deoxycholate-containing buffer, depending on the purpose for the experiment. For determination of the amount of bis-ANS bound by spectrophotometry, the LPL/bis-ANS complexes were precipitated in 50% ice cold acetone. Control experiments showed that free bis-ANS was not precipitated by this treatment.

**Fragmentation of LPL by proteinases or CNBr** - Limited proteolysis of complexes of LPL (0.3 mg/ml) and bis-ANS with trypsin or chymotrypsin was done at room temperature in 20 mM Tris-Cl, 4 mM deoxycholate, pH 8.5. After 15 min with the proteinases (1.5 μg/ml), SDS was added and the samples were heated to 95°C before analyses by SDS-PAGE (39;40). Treatment with CNBr was carried out as previously described (41).

**Determination of kinetic parameters** - The kinetic parameters $K_i$ and $A_r$ characterizing the inhibitory efficiency (explained below in the result section) were obtained from the equation:

$$A = A_r + \frac{B \cdot K_i}{K_i + C}$$

(1)

where $A$ is activity, $A_r$ is residual activity, $C$ is the concentration of bis-ANS, $K_i$ is the inhibition constant and $B$ is a proportional constant.

Fluorescence titration curves were analysed by the equation

$$F = \frac{a \cdot C}{K_d + C} + b \cdot C$$

(2)

where $F$ is fluorescence intensity, $a$ and $b$ are proportional constants and $C$ is the concentration of bis-ANS. The program SigmaPlot (SPSS Inc, Chicago, Illinois) was used for determination of the kinetic parameters.
Results

Effects of bis-ANS on the activity of LPL against different lipid substrates

Preliminary experiments showed that bis-ANS inhibited LPL both with water-soluble and with emulsified substrates. The efficiency of the inhibition was dependent on the substrate and the assay system used. There was no detectable inhibition of LPL when the assays were run in the presence of high concentrations of BSA and apoCII. In contrast, in the presence of low concentrations of BSA (< 2mg/ml), and in the absence of apoCII, inhibition was observed already at micro molar concentration of bis-ANS. These concentrations were 500-2000 times lower than the substrate concentrations used in the assay systems. The inhibition of LPL was very rapid and the kinetics was not possible to analyze by the methods used here. In the following sections we focus mainly on the LPL/bis-ANS interaction and present data obtained in systems with low concentrations of BSA (1mg/ml) and no apoCII.

The most pronounced effects of bis-ANS were observed when emulsified short chain triacylglycerols (tributyrin or tripropionin) were used as substrates (Fig. 1). The residual activity was close to zero already with 10-15 µM bis-ANS. Somewhat higher concentrations of bis-ANS were needed for inhibition of activity against the medium-chain triacylglycerol tricaprylin, the long-chain triacylglycerol triolein, or the water-soluble short-chain triacylglycerol triacetin. With p-nitrophenyl butyrate (pNPB) the activity dropped to 50 % already in the presence of 0.08 µM bis-ANS, but remained unchanged at higher bis-ANS concentrations (data not shown).

The inhibition curves could be fitted to simple decay hyperbolas, indicating that there was no cooperativity in the inhibition mechanism. Two parameters, \( K_i \) and \( A_r \), were calculated for characterization of the inhibition efficiency. The \( K_i \) values represent the apparent affinity of bis-ANS for LPL and the \( A_r \) values correspond to the residual activity of the bis-ANS/LPL complex. A high \( K_i \) value, represents a weak binding of bis-ANS to LPL. The \( K_i \) values varied more than 10 fold with different substrates. It is therefore likely that the \( K_i \) depends not only on the affinity of LPL for bis-ANS, which is probably the same in all systems, but also on the interaction of LPL with the substrate. With emulsified substrates with acyl chains containing 3, 4, 8 and 18 carbons, respectively, the \( K_i \) values increased in parallel with increasing length of the acyl chains, suggesting that bis-ANS and the substrates competed for a hydrophobic area on the enzyme. With emulsions of triacylglycerols with long acyl chains, the action of LPL occurs at the lipid/water interface in a two-dimensional heterogeneous environment where only a small fraction of the total amount of substrate and
inhibitor are accessible to the enzyme. Emulsified systems are therefore not suitable for detailed examination the inhibition mechanism. By using the soluble triacetin we were able to demonstrate partial competition between bis-ANS and individual substrate molecules (Fig. 1B). At higher triacetin concentrations, more bis-ANS was needed for the inhibition, indicating that bis-ANS impaired the substrate/LPL interaction.

The other parameter was residual activity (Ar). This characterizes the remaining activity of the bis-ANS/LPL complex. As seen in Table 1, the calculated Ar values were clearly over zero with smaller substrates like pNPB and triacetin, while for more hydrophobic substrates with longer acyl chains, the Ar values were close to zero. These results suggested that bis-ANS may sterically hinder the binding of individual substrate molecules to the active site of LPL.

Other hydrophobic probes with chemical structures similar to bis-ANS, but smaller, like ANS and T3, also inhibited LPL. Their Ki values with tributyrin as substrate were, however, much higher than those for bis-ANS (Table 2, compare with Table 1), indicating a 10 to 50-fold lower affinity of LPL for ANS and T3 than for bis-ANS. Complexes of LPL with ANS or T3 retained more activity than complexes with bis-ANS (Table 2, Ar values, compare with Table 1).

\textbf{bis-ANS had no effect on the interaction of LPL with heparin or lipid particles or on the dimeric structure of LPL.}

One possible reason for the inhibition caused by bis-ANS was impaired binding of LPL to the emulsion particles. To test this, we mixed $^{125}$I-labeled LPL with emulsified tributyrin and added increasing amounts of bis-ANS. The ratio of free/bound LPL was determined after sedimentation of the emulsion by centrifugation. We found that relatively low concentrations of bis-ANS, which were sufficient for complete inhibition of LPL activity, did not affect the binding of LPL to the lipid droplets (Table 3). However, at very high concentrations of bis-ANS (>75 µM), binding of LPL to the emulsion particles was decreased. Since we were focused mainly on the effects of the high affinity binding of bis-ANS to LPL, we did not further investigate the effects at high concentrations of the inhibitor.

Another possible explanation for the inhibition was that bis-ANS caused dissociation of active LPL dimers to inactive monomers. Ultracentrifugation in sucrose density gradients in the presence of bis-ANS at a molar excess of 2:1 or 20:1 over LPL demonstrated that the remaining LPL activity (measured against triolein), sedimented corresponding to dimeric enzyme (Fig. 2A). LPL monomers have lower affinity than LPL...
dimers for heparin and separation can therefore also be achieved by chromatography on heparin-Sepharose (42;43). We therefore investigated binding of complexes of bis-ANS and LPL to heparin-Sepharose (Fig. 2B). The peak of absorbance at 390 nm, corresponding to bis-ANS, eluted at 1.2 M NaCl and coincided with the main protein peak, detected at 280 nm, representing the elution position of active LPL dimers. Thus, in spite of containing two negatively charged sulfonyl groups, bis-ANS did not affect the interaction of LPL with heparin. Furthermore, the complexes of bis-ANS with LPL dimers persisted during the chromatography. These results supported the finding from the sucrose density centrifugation that bis-ANS did not cause dissociation of LPL to monomers. More detailed experiments, using heparin-covered sensorchips for the BIAcore, showed that the heparin affinity was the same for complexes of bis-ANS and LPL as for LPL alone (data not shown). In this system we could demonstrate that complexes of bis-ANS and LPL were able to mediate binding of lipoproteins (LDL and VLDL) to the heparin-covered sensorchips with the same efficiency as non-inhibited LPL (data not shown). These results supported the conclusion reached above in experiments with tributyrin that bis-ANS did not primarily inhibit binding of LPL to lipid/water interfaces.

**ApoCII prevented the inhibition of LPL by bis-ANS**

The presence of apoCII protected LPL from inhibition by bis-ANS and restored the inhibited activity with triacylglycerol substrates with medium (tributyrin and tricaprylin) and with long (triolein) acyl chains. In contrast, no effects of apoCII were seen when triacetin, tripropionin and pNPB were used. Thus, the reactivation by apoCII was substrate dependent. Figure 3A presents plots of restored activities as a function of apoCII concentration for triacetin, tripropionin, tributyrin and tricaprylin. Note that apoCII restored the LPL activity against tributyrin to 100 %, although it is previously known that apoCII is not needed for activity of LPL with this substrate (23). ApoCII increases the activity of LPL only with substrates containing fatty acid acyl chains longer than seven carbons (24). Our present data imply that apoCII forms a productive complex with LPL also in systems with tributyrin, but that this does not change the maximal activity of LPL unless bis-ANS is present as a competitor. Reactivation was observed at concentrations of apoCII 20-50 fold lower than the concentration of the inhibitor (8-15 µM).

To explore whether the protective effects of apoCII were related to its specific binding to LPL, we used mutants of apoCII (Y63F, Q70E and 63A insertion) that in previous studies were shown to be unable to activate LPL (16). We found that these mutants were
completely unable to restore the activity of LPL when inhibited by bis-ANS (Fig. 3B). A synthetic fragment of apoCII, spanning amino acid residues 51-79 and containing the binding site for LPL, restored about 20% of the inhibited activity. These results demonstrate that specific interaction between apoCII and LPL was needed to rescue LPL from the inhibition.

Figure 3C shows reactivation curves using tricaprylin as substrate in the presence of different concentrations of bis-ANS. At higher concentrations of the inhibitor, more apoCII was needed for reactivation, indicating competition between bis-ANS and apoCII for the same binding area on LPL.

**Studies of complex formation of LPL with bis-ANS by fluorescence measurements**

Fluorescence measurements were performed to study the formation of complexes between bis-ANS and LPL in buffer solutions without substrate. Bis-ANS alone yields only weak fluorescence with an emission maximum at 515–530 nm upon excitation at 380-390 nm and essentially no fluorescence upon excitation at 280-290 nm. The fluorescence is much increased and markedly blue shifted when bis-ANS is bound to proteins, indicating a hydrophobic nature of the protein/bis-ANS interaction. Binding of bis-ANS to proteins can also be studied by using energy transfer from tryptophanes located close to the dye. In this case the tryptophanes are excited at 280-290 nm. Figure 4 shows data of fluorescence titration of LPL with bis-ANS on excitation of tryptophanes. The high affinity binding of bis-ANS to LPL, observable at the lowest concentrations of bis-ANS, was accompanied by a marked blue shift of the emission maximum from 510 nm to 470 nm (curves 2-4). This suggested that bis-ANS was bound to a hydrophobic area on the protein. Increasing the bis-ANS concentration (from 0.5 to 2 µM) shifted the maximum emission back to 500 nm, suggesting that in addition to high affinity binding sites, there are a number of binding sites with lower affinity and hydrophobicity. The inset in Fig. 4 demonstrates how the emission maximum wavelength depended on the concentration of bis-ANS. Following addition of bis-ANS to LPL, there was a decrease in the tryptophan fluorescence emission at 342 nm, concurrent with the increase in bis-ANS fluorescence at 470-505 nm. When the interaction was followed by direct excitation of bis-ANS at 390 nm, the changes in the fluorescence emission spectra were similar to those observed by energy transfer, but the absolute values were lower (data not shown). The high affinity binding, observed by fluorescence measurements at low concentrations of bis-ANS, correlated well with the loss of LPL activity detected with tributyrin as substrate (Fig. 5). Already at equimolar concentration of bis-ANS
and LPL (0.2 µM) approximately 60-70% of the activity was inhibited. This corresponded well to a sharp increase in the fluorescence emission. Additional binding of bis-ANS to binding sites with lower affinity had little effect on the remaining activity. The apparent equilibrium dissociation constant $K_d$ calculated from the inhibition curve (equation 1) was equal to $0.13 \pm 0.02$ µM. This value was in good accordance with the $K_d = 0.10 \pm 0.02$ µM for the high affinity binding site calculated from the fluorescence curve (equation 2). The concentrations of LPL needed for fluorescence measurements were too high for direct activity measurements. Therefore the samples were diluted 100-fold (to concentrations lower than 10 nM) in the assay system. The inhibition persisted during at least 15 minutes after dilution even though the bis-ANS concentration was then diluted to levels which did not inhibit LPL on preincubation (Fig. 1). This observation demonstrated that the dissociation of bis-ANS from the high affinity binding sites on LPL was a slow process.

To study the effects of long chain fatty acids on the interaction between bis-ANS and LPL, fluorescence titration of LPL by bis-ANS was performed in the presence of oleic acid (Fig. 6). Due to the limited solubility, only low concentrations of oleic acid could be used (1 µM). LPL binds 4-6 fatty acid molecules with a $K_d$ in the order of 0.1-1.0 µM (10). If fatty acids could impair the interaction of bis-ANS with a hydrophobic site on LPL, the effect should be detectable already at this low concentration. Since the fluorescence titration curves determined in the presence and in the absence of oleic acid coincided, we concluded that the high affinity binding sites for fatty acids and for bis-ANS are most likely located in different regions of the enzyme. In contrast, the presence of 0.1 M of the soluble substrate triacetin lowered the fluorescence level, indicating that the substrate competed with bis-ANS for binding to the same area on the enzyme (Fig. 6.). This result was in accordance with the observation above that more bis-ANS was needed for the inhibition at higher concentrations of triacetin (Fig. 1B).

In a separate experiment we performed fluorescence titration of apoCII by bis-ANS. This interaction had a $K_d$ equal to 10 µM and a stoichiometry about 1:1. Thus, this interaction was found to be much weaker than the binding of bis-ANS to LPL and sequestration of bis-ANS from LPL by binding to apoCII is not likely to occur.

*Studies of complex formation of LPL with bis-ANS by surface plasmon resonance (BIAcore)*
To gain further insight into the interaction of LPL with bis-ANS we used the BIAcore system for analyses of kinetics and stoichiometry. LPL was attached to heparin, which was in turn covalently bound to the dextran matrix of the sensorchips. Solutions of bis-ANS were introduced into these sensorchips and the binding was registered as response units (RU). Response values at equilibrium, as a function of the injected bis-ANS concentrations, are shown in Figure 7. Binding studies were performed with catalytically active, dimeric LPL and for comparison also with dissociated, monomeric LPL. At low concentrations of bis-ANS, the stoichiometry for the bis-ANS/LPL complex was around 2 (calculated on 110 kDa for the LPL dimer). At higher concentrations of bis-ANS the ratio increased up to 20. If this had been a simple interaction, the dependency should follow a Langmuir-type hyperbole. However, the bis-ANS binding to active dimers of LPL did not follow this model (Fig. 7). At concentrations of bis-ANS from about 0.5 µM to 4 µM there was a shoulder in the curve, corresponding to binding of 2-3 bis-ANS per LPL dimer. The LPL activity was almost completely inhibited in this concentration range (compare with Fig. 5). The shoulder was absent when binding was studied with inactive monomers of LPL. In this case the binding of bis-ANS was very low up about to 5 µM bis-ANS, indicating that the high affinity binding site(s) was not present. From about 10 µM bis-ANS the stoichiometry for the complexes formed were similar for monomers and dimers, indicating that the low affinity binding was not dependent on structures in LPL that differed between the active dimer and the inactive monomers of LPL.

Analyses of the kinetics showed that binding at low concentrations of bis-ANS (dimeric LPL) followed a simple bimolecular binding model characterized by k_{ass}=2.0x10^4 M^{-1}s^{-1} and k_{diss}=1.2x10^{-4}s^{-1}. No available kinetic model could fit the data sufficiently well at concentrations of bis-ANS between 4 to 10 µM. However, at higher concentrations of bis-ANS the association kinetics followed a multiple binding site binding model and was characterized by slow association and very fast dissociation with corresponding rate constants k_{ass}=48 M^{-1}s^{-1} and k_{diss}=0.1 s^{-1}.

**Studies of the complexes of bis-ANS with LPL by circular dichroism and fragmentation**

Studies of circular dichroism (CD) in the far UV wavelength region showed that no changes in the spectra occurred when LPL was titrated with bis-ANS in concentrations up to 8 µM (data not shown). Thus, no detectable changes occurred in the secondary structure of LPL on binding of bis-ANS at concentrations that would cause almost full inhibition of the activity against insoluble lipid substrates. To further investigate the effect of bis-ANS we studied the
cleavage pattern on limited proteolysis of LPL with trypsin and chymotrypsin according to previous protocols (39,40). No changes in the fragmentation pattern or in the kinetics of the cleavage reactions were found when 5.4 µM bis-ANS was present in the incubation mixture containing 1.8 µM LPL compared to incubations without bis-ANS (data not shown). Thus, the high-affinity binding of bis-ANS to LPL did not cause major changes in the protein conformation.

**Binding of bis-ANS to other lipases**

For comparison we studied whether bis-ANS bound to and inhibited some other lipases. Pancreatic lipase belongs to the same triglyceride lipase family as LPL. The degree of sequence homology between the two human proteins is 35 % and the active site region is conserved (2). The bile salt-stimulated lipase is more related to simple esterases like acetylcholine esterase, and in contrast to pancreatic lipase and LPL it hydrolyses both triglycerides and cholesteryl esters (44). Studies with fluorescence titration demonstrated that both of these lipases bound bis-ANS, but with considerably lower affinity than LPL did. The K\textsubscript{d} values were 15 µM and 39 µM, respectively. Activity measurements showed that these enzymes were not influenced by the presence of bis-ANS up to 30 µM. Thus, the effects of bis-ANS on LPL are not seen with lipases in general, not even with pancreatic lipase, which is similar to LPL both in structure and in catalytic function.

**Photoincorporation of bis-ANS into LPL**

Previous studies have shown that UV irradiation causes covalent incorporation of bis-ANS into proteins (29). This prompted us to use photoincorporation to localize the high affinity binding site(s) for bis-ANS in LPL. For this, LPL was irradiated with light at 250 nm in the presence of bis-ANS, and the incorporation was monitored by visualization with UV light after electrophoresis of the samples on SDS-polyacrylamide gels. There was a continuous increase in the amount of incorporated bis-ANS with time up to 90 minutes (Fig. 8A). To quantify the amount of photoincorporated bis-ANS, the complexes were precipitated by acetone and then dissolved in 6 M guanidine chloride. The content of bis-ANS was determined from absorbance of the extracts at 390 nm and the protein concentration was determined from absorbance at 280 nm. No fluorescence or absorbance at 390 nm was observed from precipitated samples that had not been irradiated by UV light. The results indicated that UV irradiation caused irreversible incorporation of bis-ANS into LPL. Figure
8B presents the level of incorporation as a function of the concentration of bis-ANS. This dependency was not linear, indicating that bis-ANS was incorporated into binding sites with different affinities. Even at high concentrations of bis-ANS, the stoichiometry of the photoincorporation was less than 2 mol/mol (calculated on the monomer), demonstrating that the photoincorporation of bis-ANS into LPL was incomplete. The stoichiometry at low concentrations of bis-ANS, sufficient to cause almost complete inhibition (e.g. 1 μM), was less than 0.5 mol/mol of monomer. Interestingly, apoCII was completely unable to restore activity of the enzyme after photoincorporation (at 4.2 μM bis-ANS), demonstrating that the binding site was irreversibly blocked. Control experiments showed that the lipase activity was not affected by UV irradiation in the absence of bis-ANS.

To localize the binding area(s) for photoincorporated bis-ANS in LPL we used limited proteolysis of the native protein by trypsin and complete cleavage of the denatured protein by CNBr. It was previously shown that trypsin readily knicks an exposed surface loop, that presumably covers the active site, at residue Arg228, resulting in two halves of the subunit, the C-terminal half being slightly larger (39). After photoincorporation with bis-ANS, the cleavage pattern with trypsin was the same as without bis-ANS (Coomassie stained SDS-polyacrylamide gel, not shown). Unexpectedly, fluorescence was detected at almost equal intensity in both fragments (Fig. 9), even though the enzyme was mixed with bis-ANS at a molar ratio of only 1:1 (based on the monomer concentration). This indicated that, even at low concentrations, photoactivated bis-ANS was incorporated into sites in both halves of the LPL subunit. This result may be due to the relatively large size of the bis-ANS molecule, making it inappropriate for detailed localization of binding regions. Our result could also be due to binding of bis-ANS across the antiparallel LPL dimer, in a similar way as was suggested for binding of apoCII to LPL (19). After cleavage with CNBr, almost all fluorescence was associated with a 21.8 kDa fragment spanning amino acid residues 118 to 287 in the middle of the N-terminal folding domain, which contains the active site region (Fig. 9). The fragment was identified by N-terminal amino acid sequencing in 10 cycles. After complete proteolytic cleavage, either by trypsin or by endo-Glu, followed by separation by reversed phase HPLC, we were unfortunately unable to identify any smaller fluorescently labelled fragments (data not shown).

Discussion
We demonstrate that bis-ANS binds with very high affinity to LPL compared to other proteins and that the binding to LPL is accompanied by inhibition of the enzyme’s catalytic activity. Two important interactions of LPL, with apoCII and with individual substrate molecules, were affected by this binding. There are several possible mechanisms by which bis-ANS could disturb the function of LPL. The inhibitor could prevent binding of LPL to the lipid/water interface of the emulsified lipid substrate, prevent the alignment of individual substrate molecules into the active site, block the LPL/apoCII interaction or dissociate LPL into inactive monomers. Several previous studies, based on active site inhibitors and on recombinant site-specific mutants of LPL, have shown that the events of interfacial binding and interaction with individual lipid molecules can be clearly distinguished. Active site inhibitors such as tetrahydrolipstatin (THL) and hexadecylsulfonyl-fluoride (HDS) do not prevent binding of LPL to lipoproteins or to other aggregated lipids, but rather increase the enzyme’s affinity for lipid/water interfaces(34;45;46). These inhibitors do not cause monomerization of LPL. Similarly, binding of bis-ANS to LPL affected the catalytic activity without impairing the interfacial binding of LPL to lipid emulsions. In contrast to THL and HDS, bis-ANS caused only incomplete inhibition of LPL against partly soluble substrates like triacetin and p-nitrophenylbutyrate, indicating that bis-ANS did not directly hit the active site function. These substrates are smaller than the insoluble triacylglycerols which have longer acyl chains. It is reasonable to assume that bis-ANS sterically impaired the interaction of the more bulky substrate molecules with the active site and therefore caused stronger inhibition with these substrates. This assumption was supported by the observation that compounds with similar chemical structure as bis-ANS, but smaller, such as ANS and T3, did not cause complete inhibition even when long-chain triacylglycerols were used as substrates. Thus, the most likely interpretation is that binding of bis-ANS occurred to a hydrophobic region located somewhere close to the active site, but that this region is separate from the region engaged in binding of LPL to the lipid/water interface of emulsified substrates.

The low $K_d$ values for the inhibition of LPL, between 0.1 to 0.26 $\mu$M, indicated that LPL exhibits very high affinity for bis-ANS in comparison with other proteins that have been titrated with bis-ANS (35). For most proteins the $K_d$ values lie between 10 to 50 $\mu$M. High affinity binding is usually observed only when the proteins are partly unfolded into the molten globule state (47-49). In some cases bis-ANS may interact with specialized ligand-binding regions of native proteins. For example, bis-ANS interacts strongly with the ligand-binding region of several nucleotide binding proteins (50;51) and also with the T3 binding site of protein disulfide isomerase (26). Exposed hydrophobic areas at protein surfaces are usually
functionally important in ligand binding (52). The difference between the two strongly related proteins, LPL and pancreatic lipase, concerning affinity for bis-ANS suggests that the compound may recognize a functionally important area in LPL that does not exist in pancreatic lipase, e.g. the binding-site for apoCII. Pancreatic lipase was not inhibited by bis-ANS even at very high concentrations.

The stoichiometry of binding of bis-ANS to LPL indicated that there might be possibilities for several bis-ANS molecules to bind per LPL dimer, but that there are only one or two high affinity binding sites. Binding of the first two molecules of bis-ANS to LPL correlated with loss of the catalytic activity. These high affinity sites were not present on inactive LPL monomers. We conclude that the high affinity binding sites for bis-ANS are located in specialized, exposed hydrophobic region(s) that are only present in the active, dimeric conformation of LPL. Attempts to localize these sites by UV irradiation of the bis-ANS/LPL complexes to generate covalent, fluorescence-labeled fragments were only partly successful. Cleavage of native LPL in the middle by trypsin demonstrated that bis-ANS interacted with both halves of the subunit. The largest CNBr-fragment of LPL, corresponding to the central area of the molecule (residues 118-187), was labelled by the fluorescent probe. Attempts to localize the binding area(s) in greater detail were unsuccessful, probably due to the large size of bis-ANS, which make covalent interactions with this probe rather unspecific.

The most important observation in this study was that the activity of inactive complexes of bis-ANS and LPL could be restored by addition of apoCII. Our data suggest that this resurrection was caused by displacement of bis-ANS from LPL by competition for the same or a similar site by apoCII. The possibility that free apoCII formed a complex with bis-ANS and thereby released LPL from the inhibitor can be excluded because the binding affinity of apoCII for bis-ANS was orders of magnitude weaker than that of LPL (K_d = 10 µM compared to K_d = 0.1-0.26 µM). In addition, apoCII and bis-ANS formed a complex with a maximal stoichiometry of 1:1. Thus, only a minor fraction of the added apoCII was in complex with bis-ANS at the concentrations needed for reactivation. The amount of apoCII needed to restore LPL activity increased with increasing concentrations of bis-ANS, supporting the assumption that apoCII and bis-ANS competed for the same site. Furthermore, only the active, wild-type form of human apoCII was able to restore the inhibited activity, while mutants, with one residue exchanged in the putative binding site for LPL, were completely inactive. A very interesting observation was that apoCII restored the activity of LPL even with emulsified tributyrin, although no direct effect of apoCII on the catalytic activity of LPL against this substrate has previously been found. Thus, in this system
dissociation can be seen between binding of apoCII to LPL and the detailed events normally leading to increased activity. The latter are not necessary with tributyrin, since the activity of LPL in the absence of apoCII is very high. The data demonstrate that although there was no effect of apoCII on the activity, the emulsified tributyrin was able to promote folding of apoCII to the conformation that presumably allowed the proper interaction with LPL and thereby competition with bis-ANS.

The exposed hydrophobic region on LPL that bound bis-ANS appeared to be engaged also in binding of substrate molecules. This was not possible to demonstrate with the insoluble lipid substrates that formed aggregates or emulsions, but was evident with the soluble substrate triacetin. More bis-ANS was required for inhibition at higher concentrations of this substrate. Furthermore, the inhibition was less complete with long-chain triacylglycerols, like triolein, than with tributyrin. This could indicate that the longer acyl chains were able to compete more efficiently for this site than the shorter ones. Interestingly, long chain fatty acids, which are known to bind to LPL and cause a number of effects restricting the action of the enzyme (10;12;53), did not compete for binding to the same site as bis-ANS. Thus, there may exist at least three largely independent but functionally important hydrophobic sites on LPL, one for interfacial binding, one for binding of fatty acids and one for binding of apoCII and possibly also individual substrate molecules. This third site may be the most specific, since it appears to be present only on correctly folded dimers of LPL and it was not present in other lipases. Based on the present data we think that this region is exposed in water solution. We do not think that it is identical with the active site lid since bis-ANS did not disturb the cleavage by trypsin at the tip of the lid. Moreover, binding of bis-ANS to LPL was seen in buffer in the absence of a lipid/water interface. For other lipases, binding to a lipid/water interface triggers opening of the lid, and thereby exposure of hydrophobic regions on the lid which in the closed form are hidden against the entrance of the active site (54).

Recently, McIlhargey et al. reported on identification of an apoCII-binding site in LPL based on chemical cross-linking of a C-terminal fragment of apoCII to LPL in buffer solution without lipids. The labelled fragment, spanning amino acid residues 65-86, contains two regions that are not identical in hepatic lipase (residues 65-68 and 73-79). By exchange of these regions in recombinant chimeric LPL and hepatic lipase, some dependency on apoCII could be conferred to hepatic lipase, while the effect of apoCII on the LPL mutant lacking both of these regions was reduced by 60%. In accordance with previous studies, this indicated that also other regions in LPL must be important for the activation. The authors speculate that the main interaction between LPL and apoCII must be electrostatic, since the
two identified regions contained five charged residues in LPL but only one charged residue in hepatic lipase. Interestingly, the fragment labelled by bis-ANS in our study (residues 118-287) does not contain the region identified by McIlhargey et al., and we furthermore conclude that the main interaction between LPL and apoCII is most likely hydrophobic. Thus, although knowledge has been taken a few steps further, we still do not understand the details in the activation of LPL by apoCII.

In summary, by using fluorescent hydrophobic probes commonly used to study protein structure, we found evidence for an exposed, highly hydrophobic site in LPL that appears to be involved in binding of apoCII and also in recognition of individual lipid molecules. This indicates that activation by apoCII may involve improved presentation of individual substrate molecules to the active site of LPL. The hydrophobic probe bis-ANS was a strong inhibitor of catalytic activity, but did not dissociate active LPL dimers to monomers, cause any measurable changes in the secondary structure of LPL or affect binding of LPL to lipid/water interfaces.
Footnotes

Footnote to page 1:

* This study was funded by the Swedish Medical Research Council (no. 12203) and by the Estonian Science Foundation (no. 4925). AL was the recipient of a scholarship from the Swedish Royal Academy of Sciences. The costs of publication of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 Abbreviations used: ApoCII, apolipoprotein CII; ANS, 1-anilinonaphthalene-8-sulfonic acid; bis-ANS, 1,1'-bis(anilino)-4-,4'-bis(naphtalene)-8,8'-disulfonate; BSA, bovine serum albumin; LDL, low density lipoproteins; LPL, lipoprotein lipase; T3, triiodothyronine
References


Table 1. Parameters characterizing the inhibition of LPL by bis-ANS in different substrate systems

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_i$ (µM)</th>
<th>$A_r$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacetin</td>
<td>3.10±0.19</td>
<td>7.02±1.30</td>
</tr>
<tr>
<td>Tripropionin</td>
<td>0.28±0.03</td>
<td>0.12±1.30</td>
</tr>
<tr>
<td>Tributyrin</td>
<td>0.38±0.04</td>
<td>0</td>
</tr>
<tr>
<td>Tricaprylin</td>
<td>1.65±0.34</td>
<td>0</td>
</tr>
<tr>
<td>Triolein</td>
<td>4.67±0.20</td>
<td>1.50±1.43</td>
</tr>
<tr>
<td>pNPB</td>
<td>0.04±0.01</td>
<td>28 ±5</td>
</tr>
</tbody>
</table>

Table 2. Parameters characterizing the inhibition of LPL by ANS and T3 with tributyrin as substrate

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (µM)</th>
<th>$A_r$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS</td>
<td>23.1±3.4</td>
<td>14.8±3.1</td>
</tr>
<tr>
<td>T3</td>
<td>4.0±0.9</td>
<td>12.8±6.2</td>
</tr>
</tbody>
</table>
Table 3. Effects of bis-ANS on binding of LPL to emulsion droplets of tributyrin
Catalytically active, radiolabeled LPL (10 ng/ml) was incubated for 15 minutes with an emulsion of tributyrin in 0.2 M Tris-Cl, pH 7.4 in the absence and presence of various concentrations of bis-ANS. The incubation was performed with stirring under the same conditions as used for activity measurements in the pH-stat. After centrifugation (3000xg, for 5 min), the distribution of radioactivity was measured between the top phase that contained buffer and the lower phase that contained the sedimented emulsion droplets. Data are expressed as ratios of radioactivity in the supernatant (free) and in the emulsion (bound).

<table>
<thead>
<tr>
<th>Concentration of bis-ANS (µM)</th>
<th>Distribution of LPL (free/bound)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>2.0±0,3</td>
</tr>
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<td>0.4</td>
<td>2.2±0.2</td>
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<td>3.0</td>
<td>3.0±0.2</td>
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<td>8.0</td>
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<td>22.0</td>
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</tr>
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<td>42.0</td>
<td>3.2±0.1</td>
</tr>
<tr>
<td>75.0</td>
<td>7.9±0.3</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. Inhibition of LPL by bis-ANS. A. Effects with different substrates. The following substrates were used: triacetin (▼), tripropionin (■), tributyrrin (○), tricaprylin (●) and triolein (▽). All substrates were emulsified, but in contrast to the other substrates triacetin displayed a clear, homogeneous solution. The incubation mixture contained, in addition to substrate (30 mM), 0.7 mg BSA/ml, 15 IU heparin/ml and 0.15 M NaCl. Release of acids (fatty acids) was recorded on a pH-stat set at pH 8.5. The recordings were linear with time for at least 15 min with all substrates. The activity in the absence of bis-ANS was set to 100 %.

B. Competition between bis-ANS and the water-soluble substrate triacetin. LPL activity as a function of concentration of bis-ANS was measured as above, but with pure triacetin (no gum arabic) as substrate at the following concentrations: 8 mM (▲), 40 mM (●) and 70 mM (▲).

Figure 2. No effects of bis-ANS on dimeric state or on heparin affinity of LPL.

A) Sedimentation in sucrose density gradients. LPL alone (●) or mixed with a 2-fold (○) or 20-fold (▼) molar excess of bis-ANS was subjected to ultracentrifugation in sucrose gradients from 5-20 % (w/v) in the presence of the inhibitor. Data for each condition are mean values from two centrifuge tubes. Fractions are numbered from the bottom of the gradient.

B) Chromatography on heparin- Sepharose. LPL in 20 mM Tris-Cl, 4 mM sodium deoxycholate, pH 8.5 was incubated for 15 minutes at 4°C with an equimolar concentration of bis-ANS (3.3 µM, calculated on 55 kDa subunit). The incubation mixture (1 ml) was applied on a column of heparin-Sepharose and eluted by a gradient of NaCl. Fractions of 1 ml were collected. Their content of bis-ANS and protein was determined from absorbancy at 390 nm (■) and 280 nm (●), respectively.

Figure 3. Effect of apoCII on reactivation of LPL previously inhibited by bis-ANS. A) Effects of different substrates on reactivation: The substrates used were tributyrrin (▼), tricaprylin (○), tripropionin (●) and triacetin (▽). The activity measurements were performed in solutions that contained 0.7 mg BSA/ml, 15 IU heparin/ml and 0.15 M NaCl. LPL (10 nM) was mixed with substrates (30 mM) and the reaction proceeded for 10 min prior before addition of bis-ANS (8 µM final concentration). After 10 min, apoCII was added to the
incubation mixtures from a stock solution in 6 M guanidine chloride (0.55 mM). **B)**

**Reactivation by mutants/fragment of apoCII.** Same conditions as in panel A, but with the following variants of apoCII: mutants Y63F (▼) or Q70E (○); the insertion mutant 63A (●); peptide spanning residues 50-79 from the C-terminus of apoCII (▼). **C)** **Effects of apoCII at different concentrations of bis-ANS.** Emulsified tricaprylin was used as substrate (30mM). Reactivation was measured in the absence (●) as well as in the presence of 18 µM (○) or 30 µM (▼) bis-ANS. In all panels 100 % corresponds to the LPL activity measured in the absence of apoCII and bis-ANS.

**Figure 4. Fluorescence titration of LPL by bis-ANS.** Fluorescence emission spectra of LPL (0.1 µM, subunit concentration) in 20 mM Tris-Cl, pH 8.5 containing 4 mM sodium deoxycholate with bis-ANS at the indicated concentrations (1- 0 µM; 2 - 0.04 µM; 3 - 0.2 µM; 4 - 0.6 µM; 5 - 2.2 µM; 6 - 6.2 µM; 7 - 26.2 µM ). The spectra were derived by subtracting the spectrum recorded at each concentration of bis-ANS without LPL from each of the observed spectra. Measurements were performed at 25 °C with an excitation wavelength of 290 nm. The inset shows the effect of the concentration of bis-ANS on the maximal fluorescence emission wave length (λ_max).

**Figure 5. Correlation between changes in fluorescence intensity and inhibition of LPL activity by bis-ANS.** LPL (0.2 µM, subunit concentration) in 20 mM Tris-Cl, pH 7.4, containing 0.5 M NaCl and 15 IU heparin/ml was mixed with the indicated concentrations of bis-ANS at 10° C. After 5 minutes the relative fluorescence (○) was determined at the emission maximum (470-510 nm). Residual LPL activity (●) was measured using tributyrin as substrate. The sample volume was 200 µl and the total volume of the assay system was 5 ml. LPL activity in a sample without bis-ANS was set to 100 %.

**Figure 6. Effect of oleic acid or triacetin on fluorescence titration of LPL by bis-ANS.** Experiments were performed under the same conditions as described in Fig. 5, but titration was made in the presence of 1 µM oleic acid (●) or 0.1 M triacetin (○). For comparison, the titration curve for LPL without oleic acid or triacetin is shown by the dotted line.

**Figure 7. Binding of bis-ANS to dimeric and monomeric LPL as determined by surface plasmon resonance on a BIAcore.** The sensorchips contained covalently bound heparin to
which LPL was attached. Solutions of bis-ANS in 20 mM Tris-Cl, 0.15 M NaCl, pH 7.4, were injected over the sensochips and the relative response was registered at steady-state to determine the amount of bound bis-ANS. The amount of LPL was calculated on the molecular mass of the subunit (55 kDa). Experiments were carried out both with active, dimeric LPL (●, solid line) and with inactive, monomeric LPL (□, broken line).

**Figure 8. Photoincorporation of bis-ANS into LPL.** LPL (1.3 µM, calculated on subunit concentration) in 20 mM Tris-Cl, pH 7.4, containing 0.5 M NaCl, was mixed at 4° C with the indicated concentrations of bis-ANS. The mixtures were irradiated by UV light at 250 nm for the indicated times. Then the protein was precipitated by addition of ice-cold acetone to a final concentration of 50 % (v/v). After centrifugation the pellets were washed twice by cold acetone and were then lyophillized. **A) Time course for photoincorporation.** The protein pellets were dissolved in sample buffer with SDS, heated for 5 min at 95° C and then run on SDS-polyacrylamide gels (12.5 % acrylamide (w/v)). After electrophoresis, the fluorescence in the gels was visualized by a Fluoro-S detector **B) Incorporation stoichiometry.** In this case the irradiation was for 3 h. The pellets were dissolved in 6 M guanidinium chloride and the absorbancy was determined at 280 nm and 390 nm to estimate the protein and bis-ANS concentrations, respectively. The stoichiometry was calculated based on the subunit concentration of LPL.

**Figure 9. Identification of binding site(s) for bis-ANS in LPL.** Bis-ANS was photoincorporated into LPL under the same conditions as in Figure 8 at a molar ratio 1:1. After irradiation for 30 min, LPL was subjected to cleavage by trypsin or by CNBr (details in Materials and Methods). The samples were run on SDS polyacrylamide gels (12.5 % w/v). The floourograph shows two different amounts of each sample (20 and 25 µg). The last lane to the right shows a corresponding sample of LPL that had not been treated. The molecular masses were calculated from comparison with the migration of standard proteins.
Figure 1
Figure 3
Figure 4
Figure 6
Figure 8
Figure 9
bis-ANS acts as an inhibitor of lipoprotein lipase and competes for binding with apolipoprotein CII
Aivar Lookene, Liyan Zhang, Vello Tougu and Gunilla Olivecrona

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