

Binding of active and inactive forms of lipoprotein lipase to heparin

Effects of pH

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Lipoprotein lipase has been shown to bind to, be internalized by, and perhaps be transferred through, a variety of cells. These processes may involve a heparin-like cell-surface receptor and passage through acidified cell compartments. We have therefore studied effects of low pH on the binding of the lipase to heparin and on its catalytic activity. The rate of inactivation of the lipase in solution was found to increase as the pH was lowered. Addition of heparin stabilized the enzyme. Binding of active lipoprotein lipase to heparin–Sephacrose could be demonstrated at pH down to 6.5. At pH below 6, binding could not be studied directly because the lipase was too unstable in solution. Lipase bound to heparin–Sephacrose could, however, be exposed to pH 4.5 at 10°C with little loss of activity. Binding to heparin–Sephacrose also stabilized under physiological conditions (37°C, 0.15 M-NaCl, pH 5.5–7.4). Catalytically inactive lipoprotein lipase retained the ability to bind to heparin–Sephacrose. Higher concentrations of salt were needed to displace both active and inactive lipase from heparin–Sephacrose at lower pH, indicating that the affinity increased as pH was lowered. The inactive lipase was, however, displaced by lower concentrations of salt than was active lipase.

Lipoprotein lipase (reviewed by Robinson, 1970; Nilsson-Ehle *et al.*, 1980; Cryer, 1981; Quinn *et al.*, 1983; Smith & Pownall, 1984; Olivecrona & Bengtsson, 1984) acts at the luminal side of the vascular endothelium, where it is believed to be held in place by binding to heparan sulphate chains (Olivecrona *et al.*, 1977; Cheng *et al.*, 1981; Shimada *et al.*, 1981). The enzyme is synthesized in other cells in the tissue, e.g. adipocytes, and is transferred from them to the endothelium in some as-yet-unknown manner. It has been shown that the enzyme binds to, and can be internalized by, several types of cells (Friedman *et al.*, 1982; Wallinder *et al.*, 1984). This poses the question as to whether the enzyme can undergo recirculation between cell surfaces and internal cell compartments, or if internalization always leads to delivery to lysosomes. For several other ligands it has recently been shown that a decisive factor is whether the ligand dissociates from its receptor as the endosome is acidified, or if it remains bound to the receptor and thus follows it when it returns to the cell surface (reviewed by Steinman *et al.*, 1983).

We have therefore studied whether lipoprotein lipase retains its ability to bind to heparin when the

pH is lowered, and whether it retains its catalytic activity. We have also compared the affinity of active and inactive forms of the lipase for heparin.

Materials and methods

Lipoprotein lipase was purified from bovine milk by chromatography on heparin–Sephacrose as previously described (Bengtsson & Olivecrona, 1977), but the buffer used was 10 mM-Bistris/HCl, pH 6.5 (Serva, Heidelberg, Germany), instead of veronal buffer, pH 7.4.

The enzyme was iodinated by the lactoperoxidase method and re-purified by chromatography on heparin–Sephacrose as described by Wallinder *et al.* (1984). Previous studies have demonstrated that the iodinated lipase retains its catalytic activity and its ability to bind to lipid emulsions (Bengtsson & Olivecrona, 1980) and to heparin (Wallinder *et al.*, 1984). Additional evidence that the iodinated enzyme behaves similarly to the unmodified lipase is that both are cleaved by trypsin in the same way (Bengtsson & Olivecrona, 1981), that the two can be precipitated in parallel by antibodies (Olivecrona & Bengtsson, 1984) and that they disappear

in parallel on intravenous injection into rats (Wallinder *et al.*, 1984).

To prepare inactive lipase, ^{125}I -labelled lipase in 1M-NaCl/10mM-Tris/HCl, pH7.4, with 2mg of bovine serum albumin/ml was mixed with an equal volume of 7M-guanidinium chloride in 0.5M-(NH_4) $_2\text{SO}_4$ /0.2M-NaCl/10mM-Tris/HCl, pH7.4. After 18h at 4°C the guanidinium chloride was removed by a 3h dialysis against the same buffer but without guanidinium chloride. After this treatment the lipase had no detectable catalytic activity.

Lipoprotein lipase activity was assayed with Intralipid containing tri[9,10- ^3H]oleoylglycerol as the substrate (kindly given by AB Kabi-Vitrum, Stockholm, Sweden). Heat-inactivated human serum was used as the source of activator protein. The final assay system contained, in a volume of 0.2ml, 1mg of triacylglycerol, 20 μmol of NaCl, 40 μmol of Tris, 0.2mg of heparin (AB Kabi-Vitrum), 12mg of bovine serum albumin (fraction V; Sigma) and 20% (v/v) human serum. The temperature was 25°C, the pH was 8.5. The reaction was stopped and the liberated fatty acids were extracted by the method of Belfrage & Vaughan (1969), but with 0.1M-carbonate buffer. The assay time was adjusted according to the anticipated activity, so that the total amount of fatty acids liberated would be within the linear range of the assay.

The buffers used were citrate (pH3.5), acetate (pH4.5 and pH5.5), Bistris/HCl (pH6.5), Tris/HCl (pH7.5 and 8.5) and Tris/glycine (pH9.5). Heparin (stage 14; Innolex, Park Forest South, IL, U.S.A.) was purified as described by Lindahl *et al.* (1965). Heparin-Sepharose was prepared as described by Iverius (1971).

Results

Fig. 1 illustrates the effects of pH on the rate of inactivation of lipoprotein lipase, in the absence and presence of heparin. At pH7.4, about 40% of the activity was lost after 2h at 10°C in 0.5M-NaCl. At lower, or higher, pH the loss of activity became progressively more rapid (Fig. 1). Heparin stabilized the lipase at all pH values tested; above pH 6.5 it became essentially stable (Fig. 1). In the absence of heparin, the lipase lost activity more rapidly in 0.1M- than in 0.5M-NaCl (Fig. 1). With heparin present this relation was reversed: the lipase was more stable in 0.1M-NaCl. This suggests that stabilization occurred through binding to heparin, an interaction that is expected to be weakened by elevated salt concentration (Bengtsson & Olivecrona, 1977).

The enzyme could also be stabilized by binding to heparin-Sepharose (Table 1). Although more

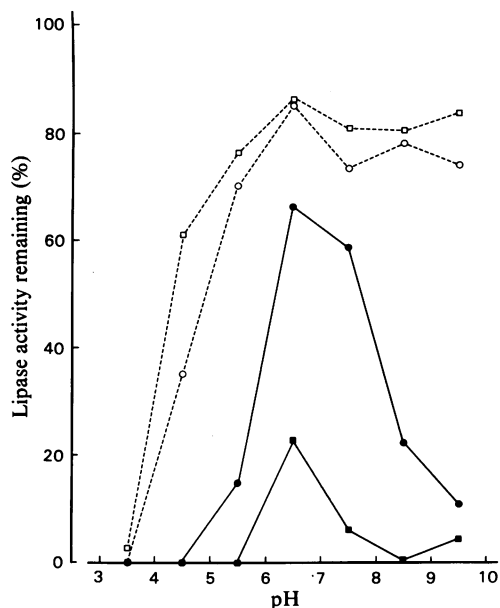


Fig. 1. Stability of lipoprotein lipase as a function of pH and salt concentration: effect of heparin

Lipoprotein lipase (0.3mg/ml in 10mM-Bistris buffer/1.2M-NaCl, pH6.5) was diluted 20-fold with buffer of the indicated pH (50mM), with (□, ○) or without (■, ●) 1mg of heparin/ml and 0.1M-NaCl (■, □) or 0.5M-NaCl (●, ○) respectively. The total volume was 200 μl and the temperature was 10°C. After 2h, 5 μl aliquots were assayed for lipase activity. The activities are expressed as percentages of the activity added. All data points represent mean values for duplicate samples.

than 80% of the lipase activity was lost during a 1h incubation in buffer at pH5.5 or 4.5, fully active lipase could be eluted from heparin-Sepharose gels that had been exposed to these buffers for the same length of time. This experiment was carried out with buffers with no salt added to maximize binding to the heparin-Sepharose. Under these conditions, dilute solutions of lipoprotein lipase are more stable than with 0.1M- or 0.5M-NaCl (Olivecrona & Bengtsson, 1984); this explains why more lipase activity was retained during incubation in buffer in this experiment than in that in Fig. 1. At 37°C in 0.15M-NaCl all enzyme activity was lost within 15min at both pH7.4 and 5.5. When lipase was bound to heparin-Sepharose at neutral pH in the cold, then exposed to 37°C, 0.15M-NaCl at pH7.4 or 5.5 for 30min, cooled and eluted with 2M-NaCl, pH7.4, 73% of the enzyme activity was recovered. Thus binding to heparin also stabilizes lipoprotein lipase at physiological salt concentration and temperature.

Table 1. Stabilization of lipoprotein lipase at low pH by binding to heparin-Sepharose

A 10 μ l portion of lipoprotein lipase (0.5mg/ml in 10mM-Bistris buffer, pH 6.5, containing 1.2M-NaCl) was incubated with 0.1 ml of heparin-Sepharose in a total volume of 1 ml of 5mM-Tris/HCl, pH 7.5, containing 0.2M-NaCl and 1 mg of bovine serum albumin/ml. After 1 h at 4°C the tubes were centrifuged (5000g, 5min) and the supernatants were removed. The gels were washed once with 0.9 ml of 50mM-Tris/HCl, pH 7.5, containing 1 mg of albumin/ml. Then 0.9 ml of 50mM-buffer of the indicated pH containing 1 mg of albumin/ml was added and the tubes were shaken for 60 min at 10°C. The supernatants were removed and their lipase activities determined. In all cases they contained less than 10% of the lipase activity originally added to the gels. The lipase remaining on the gels was then eluted by addition of 0.9 ml of 0.1 M-Tris/HCl, pH 7.5, containing 1.5M-NaCl and 1 mg of albumin/ml. After 5 min the tubes were centrifuged (5000g, 5min) and the lipase activities in the supernatants were immediately assayed. For comparison, 10 μ l of lipoprotein lipase was incubated in 1 ml of the corresponding buffers but without any gel for 65 min at 10°C. These tubes were centrifuged as described above and the lipase activities in the supernatants were determined. Results are mean lipase activities recorded in duplicate tubes.

Lipoprotein lipase activity remaining
(μ mol of fatty acids \cdot min $^{-1}$ \cdot ml $^{-1}$)

pH	In buffer	Bound to heparin-Sepharose
9.5	1.09	0.97
8.5	0.85	1.37
7.5	0.60	1.09
6.5	0.61	1.22
5.5	0.15	1.39
4.5	0.04	1.08
3.5	0.05	0.11

Fig. 2 compares the elution of lipoprotein lipase from heparin-Sepharose columns with increasing concentrations of NaCl at pH 7.4 and 6.5. Higher concentrations of NaCl were needed at the lower pH, indicating that the binding was stronger.

Lipoprotein lipase which had been unfolded in guanidinium chloride and allowed to refold in buffer was catalytically inactive, but bound almost completely to heparin-Sepharose at 0.2M-NaCl or less (Fig. 3). It was, however, displaced from the gel by lower concentrations of NaCl than was the active lipase. Thus, at 0.5M-NaCl, more than 90% of the inactive lipase, but less than 10% of the active lipase, was in the liquid phase. For this experiment 125 I-labelled lipase was used so that the distribution of inactive lipase could be easily determined. 125 I-labelled lipase that had not been

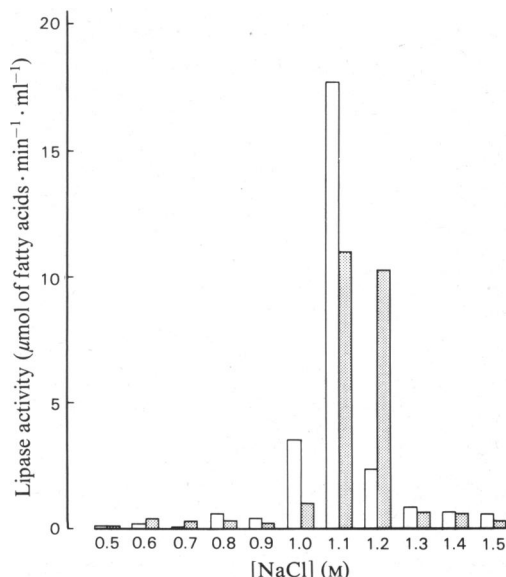


Fig. 2. Elution of lipoprotein lipase activity from heparin-Sepharose columns at pH 7.4 and pH 6.5

Two heparin-Sepharose columns (2 ml of gel, column int. diam. 10 mm) were equilibrated with 0.5M-NaCl in 10mM-Tris/HCl, pH 7.4 (open bars) and in 10mM-Bistris, pH 6.5 (hatched bars) respectively. Aliquots (400 μ l) of lipoprotein lipase (0.9 mg/ml in 10mM-Bistris, pH 6.5, containing 1.2M-NaCl) were dialysed against each of the equilibration buffers for 1 h at 10°C. A 350 μ l portion of the dialysed samples was applied to the columns and were allowed to bind for 15 min. Then the columns were eluted with 5 ml portions of the respective buffer containing the indicated concentration of NaCl. The whole experiment was carried out at 10°C and was completed within 1 h. The lipase activity in each fraction was determined immediately after its elution. The recovery of enzyme activity after the dialysis was 87% at pH 6.5 and 72% at pH 7.4. The recoveries after the heparin-Sepharose chromatography were in both cases better than 90%.

treated with guanidinium chloride gave a similar elution profile to that of active lipase. Some deviation was seen at the lower salt concentrations and was probably due to the presence of small amounts of inactive lipase in the preparation (Fig. 3).

In the experiment in Fig. 4, inactive lipoprotein lipase was allowed to bind to heparin-Sepharose at pH 7.5. Then the pH was changed as indicated and the bound lipase was eluted by successive additions of NaCl. There was a marked increase in the concentration of NaCl needed as the pH was lowered. About 0.3M-NaCl was enough to elute 30% of the lipase at pH 8.5, but more than 1M-NaCl was required at pH 4.5.

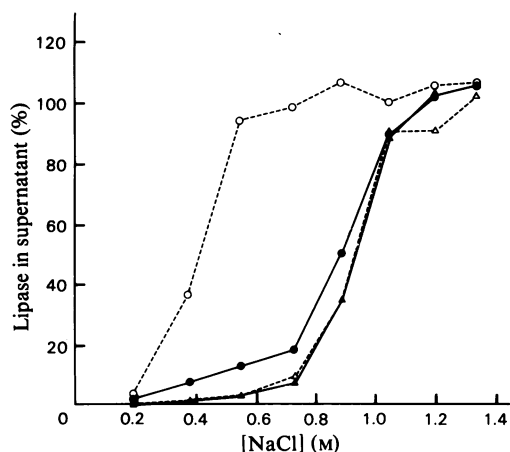


Fig. 3. Comparison of the NaCl concentration needed for elution of active and inactive lipoprotein lipase from heparin-Sepharose

A 10 μ l portion lipoprotein lipase was incubated with 0.1 ml of heparin-Sepharose and unbound lipase was removed by two rinses with 5 mM-Tris/HCl, pH 7.5, as described in the legend to Table 1. Then 0.9 ml of 50 mM-Tris/HCl, pH 7.5, containing 1 mg of albumin/ml was added. Elution of the lipase was carried out by successive additions of 40 μ l of 5 M-NaCl. After each addition the tubes were shaken for 10 min and then centrifuged (5000g, 5 min). Duplicate 10 μ l samples were taken from the supernatant for measurement of lipase activity and 20 μ l was taken for measurement of lipase radioactivity. ●, A 20 μ l portion of 125 I-labelled lipoprotein lipase corresponding to 20000 c.p.m. [in 10 mM-Tris/HCl/0.5 M-(NH₄)₂SO₄/0.2 M-NaCl, pH 7.4, with 2 mg of albumin/ml] was allowed to bind to the gel together with the unlabelled lipase (▲). ○, A 20 μ l portion of denatured 125 I-labelled lipase corresponding to 18500 c.p.m. (see the Materials and methods section) was bound, together with the unlabelled lipase (△). Thus, in both cases, data for lipase activity and lipase radioactivity were obtained from the same tube. The amounts of lipase in the supernatants are expressed as percentages of the lipase activity and radioactivity in tubes with 1 ml of 5 mM-Tris/HCl/0.2 M-NaCl, pH 7.5, containing 1 mg of albumin/ml, but without gel. The concentrations of NaCl and the amounts of lipase in the supernatants were corrected for the dilution caused by the successive addition of NaCl and the withdrawal of samples.

Discussion

The present study shows that lipoprotein lipase binds to heparin at least down to pH 4.5, i.e. at all pH values that occur in the living cell, and that the binding stabilizes the active form of the enzyme. Active lipoprotein lipase is a non-covalent dimer of two identical subunits (Iverius & Östlund-

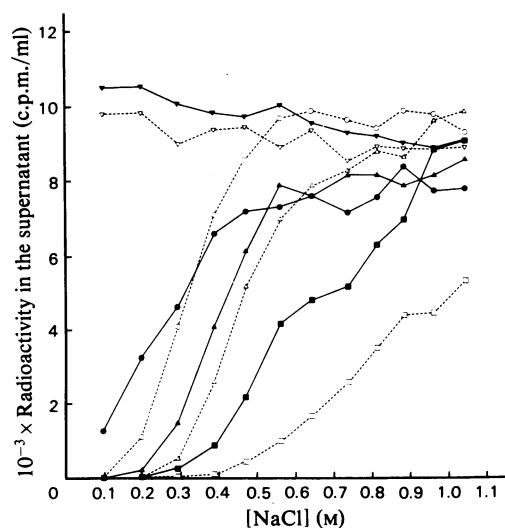


Fig. 4. pH-dependency of the affinity between inactive lipoprotein lipase and heparin-Sepharose

A 10 μ l portion of unlabelled lipase and 20 μ l of denatured 125 I-labelled lipase were bound to 0.1 ml of heparin-Sepharose in six tubes as described in the legend to Table 1. After binding, the gels were washed with 0.9 ml of 50 mM-buffer/albumin (1 mg/ml) at pH 9.5 (●), pH 8.5 (○), pH 7.5 (▲), pH 6.5 (△), pH 5.5 (■) and pH 4.5 (□) respectively. Little or no radioactive lipase was removed by this wash. Then further 0.9 ml portions of the same buffers were added and the lipase was eluted by successive additions of 20 μ l of 5 M-NaCl (as described in Fig. 3). After each addition, lipase radioactivity was measured in 20 μ l of the supernatants. Controls were made with the same amount of lipase in 1 ml of the respective buffer but without gel. They were treated as for the tubes with gel, including additions of NaCl and centrifugation. Only controls for pH 9.5 (▼) and pH 4.5 (▽) are shown. The concentrations of NaCl and the amounts of lipase in the supernatants were corrected for the dilution caused by the successive addition of NaCl and the withdrawal of samples. The unlabelled active lipase (0.3 mg/ml) was added as carrier protein in this experiment.

Lindqvist, 1976; Olivecrona & Bengtsson, 1984). After unfolding in guanidinium chloride the enzyme can regain some structure, but it remains monomeric and does not regain its catalytic activity (Olivecrona *et al.*, 1984). The inactive lipase did, however, bind to heparin-Sepharose. Thus a heparin-binding site was readily re-formed in the denatured molecule. At lower pH, quite high salt concentrations were needed to break the binding to heparin, indicating that the binding was relatively specific. Further evidence for this is that, after electrophoresis in dodecyl sulphate and

transfer to nitrocellulose, lipoprotein lipase can be identified by its ability to bind radioactive heparin (L. Socorro, A. D. Cardin & R. L. Jackson, unpublished work). Compared with active lipase, the affinity of the inactive lipase for heparin was considerably lower, as indicated by the lower salt concentration needed to elute the inactive lipase. The lower affinity for heparin could possibly be explained by structural changes in the heparin-binding site after monomerization. Alternatively, two heparin-binding sites may co-operate to establish the stronger binding of dimeric lipase to heparin-Sepharose.

In the first study on binding of lipoprotein lipase to heparin-Sepharose, veronal buffer at pH 7.4 was used (Olivecrona *et al.*, 1971). This pH has subsequently been adopted in most purification procedures that involve heparin-Sepharose. The present data suggest that a lower pH may be advantageous; the enzyme is more stable, and its affinity for heparin is higher. We now purify the enzyme at pH 6.5. The recovery is similar or better than at pH 7.4, and the stability on storage is better.

The present results indicate that, if active lipoprotein lipase bound to heparan sulphate undergoes endocytosis, it will likely remain bound and catalytically active as the endosome is acidified, and will have an excellent chance of returning to the cell surface in active form. Thus recycling, or transport through cells, of lipoprotein lipase would be possible. If, on the other hand, the lipase is internalized after binding to a receptor that allows its dissociation in the endosome, the lipase will probably rapidly lose its catalytic activity because it is unstable at low pH.

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