THE IMPORTANCE OF THE TYROSINE RESIDUES IN PANCREATIC COLIPASE FOR ITS ACTIVITY

Charlotte ERLANSON-ALBERTSSON
Department of Physiological Chemistry, University of Lund, POB 750, S-220 07 Lund 7, Sweden

Received 9 June 1980
Revised version received 30 June 1980

1. Introduction

Pancreatic colipase is secreted as a pro-colipase containing 101 amino acid residues and the N-terminal pentapeptide is lost upon tryptic cleavage, whereby the active colipase$_{96}$ is formed [1]. This smaller colipase$_{96}$ can bind to a triglyceride emulsion stabilized by phospholipids and bile salt, while colipase$_{101}$ penetrates with difficulty to reach the triglyceride. In a tributyrin-based colipase assay, the specific activity was about the same for the two forms of colipase [1]. Earlier studies have shown that the function of colipase (to bind to a bile salt-covered triglyceride substrate and to pancreatic lipase) could be localized in two different regions of the colipase molecule [2]. Binding to the triglyceride substrate was mediated by the positively charged amino-groups (from lysines) situated in the 'core' of colipase between amino acid 17 and 67, while the binding to pancreatic lipase was mediated by negatively charged carboxylic groups situated in the 'tails' of colipase either at Asp_{12}, Glu_{13} or Glu_{15} and Glu_{68} or Asp_{70} [2].

Modification of the three tyrosines of colipase, which are situated very close to each other in the primary structure [3,4]

Leu–Tyr–Gly–Val–Tyr–Tyr–Lys
53 56 57

did not affect the activity when measured on a tributyrin based assay system and the tyrosines were concluded to be unimportant for activity [2]. Spectral studies showed that the tyrosine residues were important for the interaction of colipase with taurodeoxycholate micelles [5,6]. NMR studies of the tyrosine-rich region of colipase indicated binding of bile salt molecules to this region [7–9].

Here, the importance of the tyrosine residues has been reinvestigated now using a phospholipid-covered triglyceride as substrate for colipase$_{96}$. It is shown that intact tyrosine residues are absolutely necessary for the activity of colipase in a mixed phospholipid/triglyceride based assay system, while in a pure triglyceride based assay system the tyrosine residues are not essential for the reactivation of lipase, confirming [2].

2. Materials and methods

2.1. *Materials*

Colipase$_{101}$ was prepared as in [10]. Colipase$_{96}$ was prepared from colipase$_{101}$ by tryptic hydrolysis [1]. Dextran T-500 and T-40 were obtained from Pharmacia, Uppsala and PEG-6000 from Union-Carbide, New York. Lecithin-PEG was kindly supplied by Dr G. Johansson, Dept. Biochemistry, Lund. The hydroxyl-groups of PEG were esterified with the phosphate group of lecithin.

N-Acetylimidazole was prepared in the laboratory by reaction of imidazole with acetic anhydride and was twice crystallized. Olive oil marked 'Puget' from Marseille, was used. Gum arabic dissolved at 10% (w/v) in distilled water was dialyzed before use. Intralipid (Vitrum, Stockholm) is a 20% emulsified fractionated soy bean triglyceride stabilized with egg lecithin (12 g/l).

Abbrevations: PEG, polyethylene glycol

2.2. *Assays*

Colipase activity on tributyrin was determined by titration [11]. As a long-chain triglyceride for colipase assay 0.5 ml sonicated emulsion, containing 1 ml olive oil and 2 ml 10% gum arabic solution [12], was added to 9.5 ml 2 mM Tris–maleate buffer (pH 9.0) containing 4 mM NaTDC, 150 mM NaCl and 1 mM $CaCl_2$. Using Intralipid as substrate the lag time until the reaction started was calculated as in [1].

2.3. *Modification of colipase*

Modification of the tyrosine residues of colipase$_{96}$ was performed as in [2], using *N*-acetylimidazole as modifying agent [13]. At the end of the reaction the protein was filtered through a Sephadex G-100 column in 10 mM NH_4HCO_3 buffer (pH 8.0). The extent of acetylation of the protein was measured as in [14].

2.4. *Binding studies*

The interaction of colipase with lecithin was studied in an aqueous two-phase system containing PEG in the upper phase and dextran in the lower phase [15]. To the upper phase increasing concentrations of lecithin–PEG were added and the partition coefficient K for colipase (concentration in upper phase/concentration in lower phase) determined. The difference in log K with and without lecithin–PEG was then calculated and the affinity of colipase for lecithin was expressed as $\Delta\log K$ vs [lecithin–PEG] [16]. The system contained a total concentration (in 1 ml) of 8% (w/w) dextran 40, 6% (w/w) PEG 6000, varying amounts of lecithin–PEG (fig.1), 150 mM NaCl, 5 mM Tris–maleate buffer (pH 7.0) and 1×10^{-6} M colipase. The presence of 150 mM NaCl counter-balances the positive charge of lecithin–PEG. Also the addition of a positively charged substituent on PEG, a 1,12-diamino dodecanoic acid ester of PEG did not affect the partition of colipase$_{96}$.

The effect of pH on binding was studied in the same system with and without 5 mg lecithin–PEG but with the following buffers: 5 mM sodium acetate at pH 4 and 5, 5 mM Tris–maleate at pH 6 and 7, 5 mM Tris–HCl at pH 8 and 9, 5 mM glycine–NaOH at pH 10, 11 and 12. For each pH the difference in K for partition of colipase with and without lecithin–PEG was calculated and expressed as $\Delta\log K$.

Table 1
Specific activity of colipase$_{96}$ and *O*-acetylated colipase$_{96}$ (two tyrosine residues blocked)

Substrate	Colipase$_{96}$	*O*-acetylated colipase$_{96}$
Tributyrin (4 mM NaTDC)	38 500	29 920
Olive oil (4 mM NaTDC)	6500	5060
Intralipid (4 mM NaTDC)	0.5	>60
Intralipid + 1 mM oleic acid		6.5
Intralipid + 2 mM oleic acid		4.5

The activity is expressed as μmol fatty acid released . min^{-1} . mg $protein^{-1}$ for tributyrin and olive oil. For Intralipid the activity is expressed as a lag time in minutes passing from the addition of colipase to full expression of the lipase-catalysed hydrolysis of Intralipid

3. Results and discussion

3.1. *Acetylation of colipase$_{96}$*

Acetylation of colipase$_{96}$ produced both a macromolecular and a monomeric derivative, which were separated by Sephadex G-100 filtration. Starting from 5 mg colipase$_{96}$, 3 mg was obtained as the monomeric *O*-acetylated compound. It was found homogeneous by polyacrylamide gel electrophoresis, migrating a little ahead of unmodified colipase$_{96}$. Determination of *O*-acetyl groups showed that 1.8 ± 0.1 of the tyrosine residues of colipase had been acetylated.

3.2. *Properties of O-acetylated colipase$_{96}$*

The ability of *O*-acetylated colipase$_{96}$ to activate lipase in different systems was tested and compared to unmodified colipase$_{96}$. The results are summarized in table 1. As seen the specific activity of *O*-acetylated colipase$_{96}$ was only decreased to ~80% using either tributyrin in bile salt or olive oil dispersed in gum arabic and bile salt as substrate. However, when Intralipid was used as substrate the lag time for the *O*-acetylated colipase$_{96}$ was increased to >60 min (in fact it never started) compared to a lag time of 0.5 min for colipase$_{96}$, both when present at 2×10^{-7} M.

3.3. *Binding studies on O-acetylated colipase$_{96}$*

The interaction of colipase$_{96}$ and *O*-acetylated colipase$_{96}$ determined in an aqueous two-phase system containing lecithin–PEG are given in fig.1. As seen native colipase$_{96}$ had a high affinity for lecithin–PEG

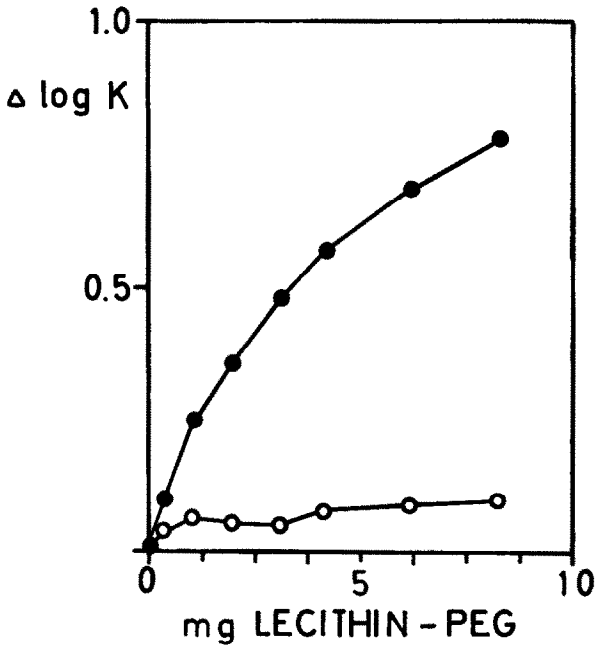

Fig.1. Change in $\Delta \log K$ for native colipase$_{96}$ and *O*-acetylated colipase$_{96}$ as a function of the amount of lecithin–PEG. Phase system: 8% dextran 40, 6% PEG 6000, varying amounts of lecithin–PEG between 0–10 mg/ml, 150 mM NaCl and 5 mM Tris–maleate buffer (pH 7.0). Native colipase$_{96}$ (•——•), *O*-acetylated colipase$_{96}$ (○——○).

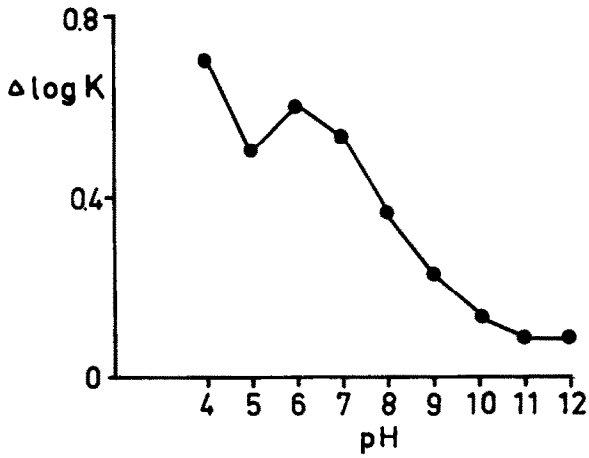

Fig.2. Effect of pH on the binding of colipase$_{96}$ to lecithin–PEG. Activity is expressed as change in $\Delta \log K$ with and without lecithin–PEG included in the PEG phase. Phase system: 8% dextran 40, 6% PEG 6000 with and without 5 mg lecithin–PEG/ml, 150 mM NaCl in 5 mM buffers at different pH values as in the text.

with an increasing value of $\Delta \log K$ at increasing concentrations of lecithin–PEG. This binding is not due to the positive charge of lecithin–PEG since no binding was observed with another positively charged PEG derivative, 1,12-diamino dodecanoic acid ester of PEG. The binding of colipase to phospholipids has been observed [17,18].

In contrast the *O*-acetylated colipase$_{96}$ had very little affinity for lecithin–PEG as $\Delta \log K$ was insignificantly affected under the same conditions. The recovery of colipase in the two phases was ~100%. Lack of binding of acetylated colipase to Intralipid was also demonstrated by binding experiments according to [19].

Thus for colipase to enable lipase to catalyze the hydrolysis of a phospholipid-covered triglyceride, binding of colipase to the phospholipids covering the surface is a pre-requisite. This binding of colipase is dependent on intact tyrosine residues.

The effect of pH showed strongest binding of colipase$_{96}$ to lecithin–PEG at pH 4 with a second optimum at pH 6 decreasing to zero at pH >10 (fig.2). The apparent pK_a values of the tyrosines of colipase were calculated to be 10.2, 10.3 and 11.8 with one of the tyrosines having properties of a 'buried' residue [9]. The reactivity of the *N*-acetylimidazole used for blocking the tyrosine residues is primarily with those tyrosines exposed on protein surfaces [20]. This agrees well with reaction of the two tyrosines not 'buried' in colipase. Furthermore, the pH dependence of the interaction of colipase$_{96}$ with lecithin indicated the involvement of the tyrosine hydroxyl groups, probably those with pK_a 10.2 and 10.3. When protonated they may form hydrogen bonds with the oxygen atoms of the phosphate group of the phospholipid molecule. Furthermore, if the two tyrosines are in close proximity in the tertiary structure and can hydrogen bond to the same phospholipid molecule, the binding should be strongly increased.

3.4. *Effect of fatty acids on the lag phase*

A 10 mM solution of oleic acid in 20 mM NaTDC (pH 8.0) was prepared. Varying amounts of this solution were added to the titration vessels containing lipase, *O*-acetylated colipase and Intralipid. The results in table 1 show that the presence of oleic acid enabled the modified colipase to start the lipase catalyzed hydrolysis of the triglycerides in Intralipid with a very short lag time. Fatty acids have been shown to bind to colipase in the presence of bile salt [21]. In

our experiments the fatty acids bound to colipase could be imagined to replace the tyrosine residues, their carboxylate groups forming hydrogen and/or ionic bonds with the phospholipids covering the triglycerides, in this way re-establishing the activity of the *O*-acetylated colipase on the lipase-catalyzed hydrolysis of Intralipid. Fatty acids will decrease the lag time of the lipase catalyzed hydrolysis of Intralipid in the presence of colipase [22].

In conclusion the tyrosine containing area of colipase is believed to form the lipid affinity site of colipase [23]. Bile salts have been shown to bind to this region [5–9] as does the non-ionic detergent Triton X-100 [23]. These experiments indicate that binding of phospholipids to colipase also involves the tyrosine residues.

Acknowledgements

Professor Bengt Borgström is thanked for fruitful discussions, Miss Ulla Johannesson for skillful technical assistance and Dr Göte Johansson for providing the lecithin–PEG. This work was supported by grants from the Swedish Medical Research Council (B79-03X-00071-15B).

References

[1] Borgström, B., Wieloch, T. and Erlanson-Albertsson, C. (1979) FEBS Lett. 108, 407–410.
[2] Erlanson, C., Barrowman, J. and Borgström, B. (1977) Biochim. Biophys. Acta 489, 150–162.
[3] Charles, M., Erlanson, C., Bianchetta, J., Joffre, J., Guidoni, A. and Rovery, M. (1974) Biochim. Biophys. Acta 359, 186–197.
[4] Erlanson, C., Charles, M., Astier, M. and Desnuelle, P. (1974) Biochim. Biophys. Acta 359, 198–203.
[5] Sari, H., Entressangles, B. and Desnuelle, P. (1975) Eur. J. Biochem. 58, 561–565.
[6] Sari, H., Granon, S. and Sémériva, M. (1978) FEBS Lett. 95, 229–234.
[7] Cozzone, P. (1976) FEBS Lett. 69, 153–156.
[8] Wieloch, T. and Falk, K.-E. (1978) FEBS Lett. 85, 271–274.
[9] Wieloch, T., Borgström, B., Falk, K.-E. and Forsén, S. (1979) Biochemistry 18, 1622–1628.
[10] Erlanson, C., Fernlund, P. and Borgström, B. (1973) Biochim. Biophys. Acta 310, 437–445.
[11] Borgström, B. and Erlanson, C. (1973) Eur. J. Biochem. 37, 60–68.
[12] Desnuelle, P., Constantin, M. J. and Baldy, J. (1955) Bull. Soc. Chim. Biol. 37, 285–290.
[13] Simpson, R. T., Riordan, J. F. and Vallee, B. L. (1962) Biochemistry 2, 616–622.
[14] Sjöholm, I., Ekenäs, A.-K. and Sjöqvist, J. (1972) Eur. J. Biochem. 29, 455–460.
[15] Albertsson, P. Å. (1978) J. Chromatogr. 159, 111–122.
[16] Shanbag, V. P. and Axelsson, C.-G. (1975) Eur. J. Biochem. 60, 17–22.
[17] Lairon, D., Nalbone, G., Lafner, H., Leonardi, J., Domingo, N., Hauton, J. C. and Verger, R. (1978) Biochemistry 17, 5263–5269.
[18] Pieroni, G. and Verger, R. (1979) J. Biol. Chem. 254, 10090–10094.
[19] Erlanson-Albertsson, C. (1980) Biochim. Biophys. Acta 617, 371–382.
[20] Riordan, J. F., Wacker, W. E. C. and Vallee, B. L. (1965) Biochemistry 4, 1758–1765.
[21] Patton, J. S., Donnér, J. and Borgström, B. (1978) Biochim. Biophys. Acta 529, 67–78.
[22] Borgström, B. (1980) Gastroenterology in press.
[23] Canioni, P., Julien, R., Rathelot, J. and Sarda, L. (1980) Lipids 15, 6–9.