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DIRECT INVOLVEMENT OF THE C-TERMINAL EXTREMITY OF PANCREATIC LIPASE (403-449) IN COLIPASE BINDING

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After a selective cleavage of a lipase / colipase cross-linked complex, the colipase has been shown to be bound to a 5 kDa lipase fragment identified as the C-terminal extremity of the chain extending from residue 403 to the C-terminus (Cys 449). The colipase binding site on lipase is therefore localized in a restricted contact area. Moreover, from sequence comparison of lipase from various species, an acidic residue, Glu 440, is likely to be involved in ion pairing with colipase.

Pancreatic lipase (50 kDa) (EC 3.1.1.3) plays a key role in fat digestion in the intestine by converting insoluble triacylglycerols into more polar products, fatty acids and 2-monoacylglycerides, able to cross the brush border membrane of enterocytes. Because of the insolubility of its substrate, lipase realizes an heterogeneous catalysis, the first step of which is the adsorption of the enzyme at the water / lipid interface. However, since this step is impaired by the presence of bile salt (1, 2), the pancreas synthesizes and secretes a small protein, colipase (10 kDa) (3, 4) to counteract the inhibitory effect of bile salt. The function of colipase is to anchor lipase on the bile salt coated interface (5, 6). Thus, fat digestion proceeds through protein / protein interactions mediated by an organized lipid phase (for a review, see 7).

The presence of two binding sites has been postulated on both lipase and colipase, an interfacial binding site and a colipase (or lipase) binding site. Yet, except for the colipase interfacial binding site, which has been shown to involve the tyrosine rich region of the molecule (8-13), the other sites have not been identified, despite extensive studies including the elucidation of the three-dimensional structure of human lipase (14).

As first postulated by Bousset-Risso *et al.* (15), the lipase molecule is made up of two domains. The N-terminal domain (residues 1-335), which bears the active site is separated from the C-terminal one (residues 336-449) by a strangling region (14). The two-domain organization of the enzyme has been correlated to two specific functions (16). The N-terminal domain is devoted to catalysis, whereas the C-terminal domain is responsible for colipase binding, as suggested by Mahe-Gouhier (17). In this respect, the two domains can be expected to bear the

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interfacial binding site and the colipase binding site respectively. However, no information is available concerning the precise localization of these sites.

The residues involved in the interfacial binding site of lipase could not be identified from the crystallographic data, since this site is not likely to be preformed in the molecule in solution. Actually, in the crystal, the active site is buried inside the molecule by a special folding of the chain, forming a flap. In the presence of a lipid interface, the repositioning of the flap, which is thought to be the structural basis of the interfacial activation of lipase, would create the appropriate interfacial binding site.

The lipase / colipase binding has been reported to involve both hydrophobic interactions (18) and ion pairing (19) between negatively charged residues of colipase (19) and probably lysyl residues of lipase (20). The lipase binding site on colipase is still unidentified and, although ion pairing has been shown to occur in the C-terminal domain of lipase (16), the corresponding site on lipase has not yet been localized.

The very high affinity between lipase and colipase in the presence of a lipid interface (estimated to at least 10^{10} M) is several orders of magnitude lowered down (10^{6} M) in the absence of the interface (6, 21), suggesting that, in solution, only a "minimal" binding occurs between both partners. This minimal binding still involves ion pairing, as evidenced by the obtention of a specific lipase / colipase covalent complex by cross-linking in the presence of carbodiimides (22).

The identification of the cross-linked residues in the lipase / colipase complex will be of importance in the localization of the binding sites on both proteins. However, this requires the isolation of all the cross-linked peptide(s) which is an hazardous and time consuming work due to the size of the complex. We therefore decided to focuse, at first, our attention on the colipase binding site of lipase.

In this paper, we report that the selective chemical cleavage of lipase cross-linked to colipase, allowed us to demonstrate that colipase is cross-linked to the lipase sequence extending from residue 403 to the C-terminus (Cys 449) of the chain. Based on sequence comparison, the involvement of two lipase residues in ion pairing with colipase is discussed.

MATERIALS AND METHODS

Materials

N-cyclohexyl-N'-2-morpholinoethyl-carbodiimide-methyl-p-toluolsulfonate (CMC-CDI) and tributyrin were obtained from Fluka (Switzerland). Sodium taurodeoxycholic acid, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium and goat anti-rabbit IgG labelled with alkaline phosphatase were from Sigma (MO, USA). Ultrogel AcA 54 was from IBF (France). Fluorotrans PVDF sheet was from Pall (Poly Labo, France). Red ponceau was from Serva (France).

Methods

Protein purification

Homogeneous porcine pancreatic colipase was obtained from fresh pancreas according to Chapus *et al.* (23). Horse pancreatic lipase was purified from pancreatic delipidated acetone powder following the process described by Lombardo *et al.* (24). Protein concentrations were determined at 280 nm using a molar absorption coefficient of 0.4×10^4 M^{-1} cm^{-1} and 6.65×10^4 M^{-1} cm^{-1} for porcine colipase and horse lipase respectively.

Cross-linking reaction

Horse pancreatic lipase and porcine colipase were cross-linked as previously described using CMC-CDI as the cross-linking reagent (22). The complex was isolated by molecular sieving on

Ultrogel AcA 54 as reported (22). The isolated complex was completely devoid of any contamination by colipase but still contained traces of free lipase.

Cleavage of tryptophanyl bonds in lipase and cross- linked complex.

Cleavage of the horse pancreatic lipase (20 nanomol) and of the horse lipase / porcine colipase cross-linked complex (30 nanomol) was achieved as indicated by Fontana *et al.* (25). Iodozobenzoic acid, at a tryptophane / reagent molar ratio of 1/50, was preincubated for 2 hours with p-cresol (molar ratio tyrosine / p-cresol = 1/20) in a guanidinium buffer, in order to avoid cleavage of the tyrosyl bonds. The reduced and carboxymethylated proteins were then added and incubated for 24 h or 48 h in the dark at room temperature. The reaction was stopped by addition of mercaptoethanol.

Gel electrophoresis

Slab gel electrophoresis in the presence of SDS was performed according to Laemmli (26). After staining with Coomassie brilliant blue, slabs were destained using an acetic acid/ethanol/water mixture (10:30:60, vol/vol).

Western blotting

Western blots were performed according to Burnette (27). The peptides generated by chemical cleavage of lipase free or cross-linked to colipase were loaded in duplicate on a 12 % polyacrylamide slab and separated by electrophoresis in the presence of SDS. After transfer on a PVDF sheet, the two lanes were cut apart, the first one for immunodetection, the second one being saved for subsequent analysis. The first lane was incubated with specific antibodies directed either against lipase or colipase. Immunodetection was then performed with alkaline phosphatase labelled goat anti-rabbit IgG after incubation in a 0.1 M Tris-HCl buffer containing 0.1 M NaCl, 1 mM $MgCl_2$, $5x10^{-4}$M BCIP and nitro blue tetrazolium, pH 9.5. The second lane was transitorily revealed using ponceau red.

The cross-linked peptides were localized in the second lane by juxtaposition of the two lanes and cut off from the PVDF sheet. The stripes of PVDF sheet were then submitted to microsequencing, after destaining with water.

N-terminal sequence analysis.

N-terminal sequence analyses were performed by stepwise Edman degradation using a gas-phase sequencer (Applied biosystems, model 470 A). The resulting phenylthiohydantoins were analyzed by HPLC using a C18 column (Brownlee, 5 µm, 2.1 x 220 mm). They were eluted using a gradient from 10 to 46 % methanol in a 7 mM sodium acetate buffer, pH 4.84.

RESULTS AND DISCUSSION

The cross-linking reaction between lipase and colipase has been performed using carbodiimides in order to convert ion pairing occuring between charged residues of the two partners into covalent linkage (22). The strategy usually adopted to identify cross-linked peptides was the following:

(i) preparation of the cross-linked complex.

(ii) cleavage of the complex.

(iii) isolation and characterization of the cross-linked peptides.

In order to make the identification of lipase/colipase cross-linked peptides easier, we decided to use intact colipase as a marker of these peptides. Therefore, based on the ability of lipase and colipase from various species to specifically interact (28, 29), a cross-linked complex associating the horse lipase with the porcine colipase was prepared. Porcine colipase being devoid of tryptophanyl residues, the cleavage of tryptophanyl bonds in the complex can be expected to yield lipase fragments free or cross-linked to the intact colipase molecule. The latter peptides can be easily detected by immunodetection using anticolipase antibodies.

1) Isolation of the cross-linked peptides.

A comparative study of the cleavage of tryptophanyl bonds in the horse lipase either free or cross-linked to porcine colipase was performed as described in Materials and Methods. The

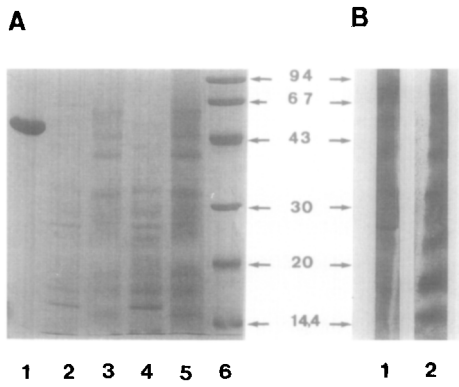

Fig. 1. *Cleavage of tryptophanyl bonds in lipase either free or cross-linked to colipase.* The cleavage was performed by incubation of the protein samples with iodosobenzoic acid as described in Materials and Methods.
(A) Patterns of cleavage revealed by SDS electrophoresis. Native lipase (lane 1); 24 h incubation of lipase either free (lane 2) or cross-linked to colipase (lane 3); 48 h incubation of lipase either free (lane 4) or cross-linked to colipase (lane 5); molecular mass markers (lane 6).
(B) Immunodetection of the peptides of lane 5 (Fig.1A) using either antilipase (lane 1) or anticolipase (lane 2) antibodies.

peptides thus generated were separated by electrophoresis in the presence of SDS. As shown in figure 1A, different patterns of cleavage were obtained with lipase either alone (lanes 2 and 4) or cross-linked to colipase (lanes 3 and 5). The differential peptides in the latter case were expected to contain colipase.

In order to check this assumption, immunodetection using either antilipase or anticolipase antibodies was carried out on the fragments resulting from lipase or the cross-linked complex. In the first case, the fragments were detected only with antilipase antibodies (data not shown), whereas in the second case, among the peptides detected by antilipase antibodies (fig. 1B, lane 1), some of them gave also a positive signal with anticolipase antibodies (fig. 1B, lane 2). This result indicates that the cleavage of tryptophanyl bonds in the complex yielded both lipase fragments either free or cross-linked to colipase, as expected.

A special attention was paid to the cross-linked peptides of low molecular mass in order to characterize the smallest lipase fragment involved in the colipase binding. Actually, since intact colipase has a molecular mass of 10 kDa, the size of the lipase fragment(s) associated to colipase could be estimated. Thus, a peptide of 15 kDa, which was the smallest one shown to react with both antilipase and anticolipase antibodies, was selected and submitted to microsequencing.

2) Analysis of the selected 15 kDa cross-linked peptide.

Automated N-terminal sequencing of the 15 kDa peptide yielded the following sequence:

Tyr-Asn-Asn-Val-Ile-Asn-Leu-Thr-Leu-Pro -

This sequence was easily identified to the N-terminal sequence of the fragment 403-449 (30) generated by the cleavage of the Trp402-Tyr403 bond in the lipase chain. The sum of the molecular mass of the lipase fragement (5 kDa) and that of colipase (10 kDa) is in fairly good agreement with that of the cross-linked peptide. However, surprisingly the N-terminal sequence of colipase was not detected. Considering the molecular mass of this peptide and its positive detection with anti-colipase antibodies, the most likely explanation was that the N-terminal residue of the cross-linked colipase was blocked. This is in agreement with the fact that the N-terminal

Table 1

Alignement of the sequence extending from residue 402 to the C-terminus in pancreatic lipase from various species

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      410        420        430        440       449
WYNN-VINLTLPKVGASKITVER-NDGSVFNFCSEETVREDVLLTLTAC        Horse (30)
WYNNNVINPTLPRVGASKITVER-NDGKVYDFCSQETVREEVLLTLNPC        Pig   (31)
WYNN-VINPTLPRVGASKIIVET-NVGKQFNFCSPETVREEVLLTLTPC        Human (32)
WNNN-VVNPTFPKVGAAKITVQKGEEKTVHSFCSESTVREDVLLTLTPC        Dog   (33)
WYNN-VINPTLPKVGASRISVER-NDGRVFNFCSQDTVREDVLLTLSAC        Rat 1 (34)
WNNQ-VINPSFPKVGAAKITVQKGEERTEYNFCSEETVREDTLLTLLPCTESDTV  Rat 2 (35)
   N-VINPTLPKVGASKITVE                  EEVLLTLNPC        Sheep (36)
                    ↑                       ↑
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The numbering system of the porcine lipase has been used throughout. Several gaps (-) have been introduced in order to optimize sequence alignments. Lys 419 and Glu 440 are indicated by arrows.

sequencing of the intact cross-linked complex yielded only the N-terminal sequence of lipase, definitely proving that the N-terminal residue of colipase was blocked during the cross-linking reaction probably because of an intramolecular side-reaction. This result shows that the fragment 403-449 is directly involved in colipase binding in solution and contains the residue(s) participating in the ion pairing with colipase. This conclusion can be widened to the binding between the two partners at the interface since, as previously reported (16), lipase depleted of the C-terminal fragment 410-449 can no longer be cross-linked to colipase, nor reactivated by colipase. This strongly suggests that the C-terminal extremity of the lipase chain bears the major part of the colipase binding site.

Since lipase and colipase from different species can specifically interact (28, 29), it can be expected that their respective binding sites, including the residues involved in ion pairing, are conserved. The comparison of the sequence extending from residue 402 to the C-terminus (Cys 449) in pancreatic lipase from various species (Table 1) focused our attention on two charged residues, Lys 419 and Glu 440. Glu 440, is strictly conserved among all lipases. By contrast, Lys 419, although present in most species, is replaced by an arginine in one form of rat lipase (34) which has been found to be reactivated by colipase from different species (29). Although previous studies reported that lysyl residues of lipase are likely to be involved in colipase binding, it cannot be ruled out from our observations that ion pairing occurs between a carboxylic group of lipase (Glu 440) and the ε-amino group of Lys 24, the only conserved lysine in colipase. In this respect, it must be pointed out that all previous studies were performed in the presence of an interface that is known to strongly increase the affinity between lipase and colipase. Therefore, it can be assessed that the minimal binding which occurs in solution between lipase and colipase and involves Glu 440 and Lys 24 , is strenghtened in the presence of an interface probably because of the involvement of a greater number of either hydrophobic or (and) ion pairing interactions.

Moreover, the involvement of both Lys 419 and Glu 430 in the colipase binding site is unlikely since the recent elucidation of the three dimensional structure of the horse lipase at 2.3 Å resolution clearly shows that the two residues are located on opposite sides of the molecule (Y. Bourne, personnal communication).

In this work, we demonstrated for the first time that an essential ion pairing between lipase and colipase occurs at the C-terminal extremity of the lipase chain (residues 403-449).This finding

together with previous results (16) strongly suggests that, in solution, the interactions between the two partners are limited to a restricted contact area, which is likely to be extended in the presence of an interface. Moreover, from sequence comparison, Glu 440 is likely to be involved in the colipase binding site on lipase.

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REFERENCES

1. Baskys, B., Klein, E. and Lever, W. F. (1963) Arch. Biochem. Biophys. 102, 201-209.
2. Morgan, R. G. H., Barrowman, J. and Borgström, B. (1969) Biochim. Biophys. Acta 175, 65-75.
3. Maylie, M. F., Charles, M., Gache, C. and Desnuelle, P. (1971) Biochim. Biophys. Acta 229, 286-289.
4. Borgström, B. and Erlanson, C. (1971) Biochim. Biophys. Acta 242, 509-513.
5. Chapus, C., Sari, H., Sémériva, M. and Desnuelle, P. (1975) FEBS Lett. 58, 155-158.
6. Momsen, W. E. and Brockman, H. L. (1976) J. Biol. Chem. 251, 384-388.
7. Chapus, C., Rovery, M., Sarda, L. and Verger, R. (1988) Biochimie 70, 1223-1234.
8. Borgström, B. (1977) Biochim. Biophys. Acta 488, 381-391.
9. Sari, H., Granon, S. and Sémériva, M. (1978) FEBS Lett. 95, 229-234.
10. Canioni, P. and Cozzone, P. (1979) Biochimie 61, 343-354.
11. Cozzone, P.J., Canioni, P., Sarda, L. and Kaptein, R. (1981) Eur. J. Biochem. 114, 119-126.
12. De Caro, J. D., Behnke, W. D., Bonicel, J., Desnuelle, P. and Rovery, M. (1983) Biochim. Biophys. Acta 747, 253-262.
13. Granon, S. (1986) Biochim. Biophys. Acta. 874, 54-60.
14. Winkler, F. K., D'Arcy, A. and Hunziker, W. (1990) Nature 343, 771-774.
15. Bousset-Risso, M., Bonicel, J. and Rovery, M. (1985) FEBS Lett. 182, 323-326.
16. Abousalham, A., Chaillan, C., Kerfelec, B., Foglizzo, E. and Chapus, C. (1992) Protein Engineering 5, 105-111.
17. Mahe-Gouhier, N. and Leger, C. (1988) Biochim. Biophys. Acta 962, 91-97.
18. Mahe, N., Leger, C., Linard, A. and Alessandri, J. M. (1987) J. Chromatogr. 395, 511-521.
19. Erlanson, C. (1977) FEBS Lett. 84, 79-82.
20. Erlanson, C., Barrowman, J. A. and Borgström, B. (1977) Biochim. Biophys. Acta 489, 150-162.
21. Patton, J. S., Albertsson, P. A., Erlanson, C. and Borgström, B. (1978) J. Biol. Chem. 253, 4195-4202.
22. Chaillan, C., Rogalska, E., Chapus, C. and Lombardo, D. (1989) FEBS Lett. 257, 443-446.
23. Chapus, C., Desnuelle, P. and Foglizzo, E. (1981) Eur. J. Biochem. 115, 99-105.
24. Lombardo, D., Chapus, C., Bourne, Y. and Cambillau, C. (1989) J. Mol. Biol. 205, 259-261.
25. Fontana, A., Dalzoppo, D., Grandi, C. and Zambonin, M. (1981) Biochemistry 20, 6997-7004.
26. Laemmli, U. K. (1970) Nature 227, 680-685.
27. Burnette, W. N. (1981) Anal. Biochem. 112, 195-203.
28. Rathelot, J., Julien, R., Bosc-Bierne, I., Gargouri, Y., Canioni, P. and Sarda, L. (1981) Biochimie 63, 227-234.
29. Sternby, B. and Borgström, B. (1981) Comp. Biochem. Physiol. 68B, 15-18.
30. Kerfelec, B., Foglizzo, E., Bonicel, J., Bougis, P. E. and Chapus, C. Submitted.
31. De Caro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P. and Rovery; M. (1981) Biochim. Biophys. Acta 671, 129-138.
32. Lowe, M. E., Rosenblum, J. L. and Strauss, A. W. (1989) J. Biol. Chem. 264, 20042-20048.
33. Kerfelec, B., Laforge, K. S., Puigserver, A. and Scheele, G. (1986) Pancreas 1, 430-437.
34. Sims, H. F., Strauss, A. W. and Lowe, M. E. (1991) Genbank, M58369.
35. Wicker-Planquart, C. and Puigserver, A. (1992) FEBS Lett. 1, 61-66.
36. Gieseg, S. P., Grigor, M. R and Carne, A. (1991) Biochemistry International 23,949-957.