

# A cross-linked complex between horse pancreatic lipase and colipase

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The water soluble carbodiimide *N*-cyclohexyl-*N*'-2-morpholinoethyl-carbodiimide-methyl-*p*-toluolsulfonate was found to effectively covalently cross-link pancreatic colipase to lipase as evidenced by Western blotting experiments using antibodies directed either against lipase or colipase. Moreover the resulting covalent complex has a *M*<sub>r</sub> consistent with a stoichiometry of 1 mol colipase per mol lipase. Cross-linked lipase and colipase retain their activity implying a correct covalent binding between the two proteins. The specificity of the lipase-colipase binding was further supported by the very low amount of cross-linked products when lipase or colipase alone were incubated in the presence of carbodiimide. The formation of a covalent lipase-colipase complex in the presence of carbodiimide clearly demonstrates that the binding between both proteins involves ion pairing. Furthermore, the formation of an active covalent complex strongly suggests that the lipase-colipase binding site is distinct from the colipase interfacial recognition site as well as from the lipase catalytic site.

Lipase; Colipase; Complex; Crosslinking

## 1. INTRODUCTION

Since pancreatic lipase normally acts at triglyceride-water interfaces, interfacial adsorption of the enzyme can be expected to be an essential step in catalysis. Lipase action on emulsified substrates has been shown to be inhibited by the presence of bile salts [1,2]. This inhibitory effect can be counteracted by a small pancreatic protein, the so-called colipase [1–4]. Colipase was assumed to anchor lipase on the bile salt-coated lipid interface [4]. It was also found to be able to stabilize lipase against interfacial denaturation [5]. These functions require specific protein-protein interactions mediated by the presence of an organized lipid interface. The existence of two distinct binding sites on colipase has been postulated; an interfacial binding site and a lipase binding site. As a consequence, a colipase binding site complementary of the above-mentioned lipase binding is likely to occur on lipase.

While the interfacial binding site of colipase, which is likely to involve the tyrosine-rich region of the molecule, has been extensively studied [6–10], the lipase binding site of colipase as well as the colipase binding site of lipase are not as well documented. Nevertheless, the specific lipase-colipase binding would involve ion pairing between negatively charged residues

of colipase [11] and probably lysyl residues of lipase [12].

The purpose of the present work was to confirm ion pairing interactions in the binding of colipase to lipase, through the formation of a specific covalent binary complex using a water soluble carbodiimide.

## 2. MATERIALS AND METHODS

### 2.1. Materials

CME-CDI was obtained from Fluka (Switzerland). NaTDC, BCIP, nitro blue tetrazolium and goat anti-rabbit IgG labelled with alkaline phosphatase were from Sigma (USA). Molecular sieve Ultrogel AcA 54 was a product from IBF (France). Nitrocellulose sheet (pore size = 0.45 µm) and filter paper were purchased from Schleicher and Schuell (FRG).

### 2.2. Protein purification

Homogenous colipase was obtained from fresh horse pancreatic gland according to Chapus et al. [13]. Horse lipase was purified from pancreatic delipidated acetone powder following the process described by Lombardo et al. [14].

### 2.3. Protein concentration

Colipase and lipase concentrations were determined at 280 nm using  $E_{1\%}^{1\text{cm}}$  = 8.0 and 13.3, respectively.

### 2.4. Activities measurements

Lipase activity was assayed titrimetrically as reported [15] using 0.11 M emulsified tributyrin in a 1 mM Tris-HCl, pH 7.4 buffer containing 0.1 M NaCl, 5 mM CaCl<sub>2</sub> and 1 mM NaTDC in the presence of a saturating amount of colipase. Colipase was assayed as previously described [13]. The lipase and colipase activities of the cross-linked complex were measured on tributyrin in the absence of added free lipase and colipase.

### 2.5. Cross-linking reaction

Pancreatic lipase ( $7 \times 10^{-5}$  M) and colipase (2- to 5-fold molar excess) in a 0.1 M Mes buffer, pH 6.0, containing 10 to 240 mM NaCl,

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*Abbreviations:* CME-CDI, *N*-cyclohexyl-*N*'-2-morpholinoethyl-carbodiimide-methyl-*p*-toluolsulfonate; NaTDC, sodium taurodeoxycholate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate

were incubated at 25°C with 30–180 mM CME-CDI. The reaction was stopped by adding sodium acetate to a 0.1 M final concentration. Finally, the pH of the medium was raised to pH 9.0 by addition of Tris-HCl, pH 9.0 (0.3 M final concentration).

## 2.6. Gel electrophoresis

Slab gel electrophoresis in the presence of SDS was performed according to Laemmli [16]. After Coomassie brilliant blue staining, slabs were destained using an acetic acid/ethanol/water mixture (10:30:60, v/v).

## 2.7. Western blotting

Western blots were performed according to Burnette [17]. After electrophoretic transfer of the proteins fractionated by 10% acrylamide SDS gel-electrophoresis, nitrocellulose sheets were first incubated with specific antibodies directed either against lipase or colipase. These antibodies were raised in rabbit as previously described [18]. Immunodetection was then done using alkaline phosphatase-labelled goat anti-rabbit IgG. The nitrocellulose replica was incubated for 60 min with anti-rabbit IgG, then washed as described [17] and incubated in 0.1 M Tris-HCl, pH 9.5, buffer (0.1 M NaCl, 1 mM MgCl<sub>2</sub>,  $5 \times 10^{-4}$  M BCIP and nitro blue tetrazolium).

## 2.8. Determination of the percentage of cross-linking

The percent of cross-linking was estimated from analysis of SDS electrophoresis gels performed on aliquots of the incubation mixture withdrawn at various times. After staining, the gels were scanned using an LKB 2202 ultrascan Laser Densitometer interfaced with an HP 3390A integrator. The percent of cross-linking ( $P$ ) was expressed as:

$$P = \frac{[(\text{area cross-linked complex})/(\text{area cross-linked complex} + \text{area free lipase})] \times 100.$$

# 3. RESULTS

## 3.1. Formation of a cross-linked complex between lipase and colipase

As shown in fig.1, incubation of lipase and colipase in the presence of 48 mM CME-CDI yielded a major

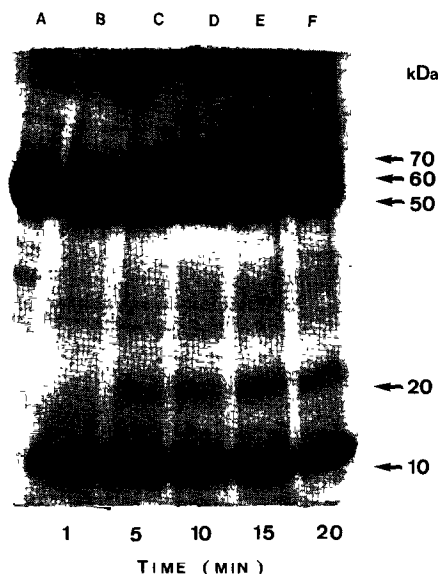


Fig.1. SDS polyacrylamide electrophoresis pattern of colipase to lipase cross-linking. Reaction as a function of time (for experimental conditions, see the text). Lane A, lipase alone; lanes B–F, analysis of the incubation medium at various times as indicated.

product with an  $M_r$  of 60000. Fig.1 also illustrates the typical time course of the cross-linking reaction. The amount of the 60 kDa molecular species increases with time, while the free lipase (50 kDa) and colipase (10 kDa) amounts decrease. The molecular mass of the cross-linked species strictly corresponds to that of a binary complex involving 1 mol lipase per mol colipase. Moreover, the Western blot presented in fig.2, unambiguously shows that the 60 kDa species contains lipase and colipase since it is recognized by specific antibodies directed either against lipase or colipase.

Control experiments of self-association performed on lipase ( $4 \times 10^{-5}$  M) or colipase ( $2 \times 10^{-4}$  M) alone yielded a very low amount of cross-linked products but never any 60 kDa molecular species. The minor bands observed in fig.1 for the long incubation times are likely to correspond to non-specific self association of 2 colipase molecules and of a lipase molecule with two or more colipase molecules. These 'non-specific' complexes can also be detected as minor contaminants in fig.2. The amount of cross-linked complex was estimated by densitometry as a function of time and was directly related to the decrease of the amount of free lipase as shown in fig.3.

The influence of several parameters, including pH, temperature, protein concentration, salt concentration, cross-linker concentration and the presence of NaTDC were tested. The cross-linking reaction was insensitive to the pH (from 6 to 7), to the NaCl concentration (from 10 mM to 240 mM) and was slightly decreased in the presence of NaTDC at micellar concentrations (2 mM). However, no cross-linking between lipase and colipase was observed at low temperature (4°C). The yield of cross-linking was definitely higher when colipase was used in slightly molar excess (4-fold) relative to lipase. When the cross-linker concentration was highly increased (from 30 to 180 mM), the incubation

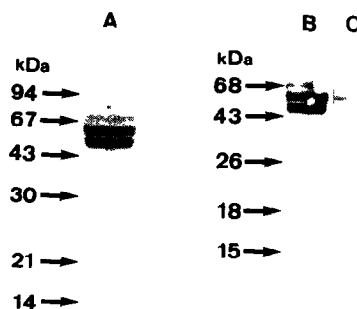


Fig.2. Analysis of the cross-linked products by Western blotting. The cross-linking conditions were those described in the text. Analysis of the medium was performed by SDS polyacrylamide electrophoresis after 10 min incubation. Lane A, Coomassie blue staining; lane B, visualization with alkaline phosphatase-labelled anti-rabbit IgG after incubation of the nitrocellulose transferred protein with specific antibodies directed against lipase; lane C, same as lane B but using antibodies against colipase.

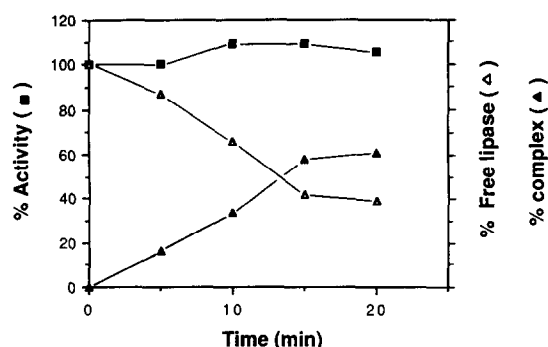


Fig. 3. Yield of colipase to lipase cross-linking and determination of lipase activity versus time during incubation. (Δ) Free lipase; (▲) cross-linked complex; (■) percent of tributyrin hydrolysis. For conditions, see the text.

medium became turbid, probably due to the formation of insoluble high molecular weight aggregates.

The amount of cross-linked complex has never exceeded 60%. The equilibrium between lipase and colipase [3,19] cannot be driven to completion because of nucleophilic water competition during the reaction.

### 3.2. Activity of cross-linked colipase-lipase complex

Concomitantly to the determination of the yield of cross-linking, the lipase activity was tested as a function

of time during the reaction. The results are also presented in fig. 3. No loss of lipase activity, within experimental error, was observed even when 60% of the free lipase had disappeared, implying that the cross-linked lipase retained its full activity.

### 3.3. Preliminary attempts of purification of the cross-linked complex

Attempts using ion exchange chromatography failed to separate the cross-linked complex from free lipase and colipase. Although the cross-linked complex and the free lipase possess close  $M_r$  values, they can be separated, to some extent, by molecular sieving. After extensive dialysis against distilled water and lyophilization, the incubation medium was loaded onto an AcA 54 Ultrogel column equilibrated with 10 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl. The elution was performed using the same buffer. As shown in fig. 4, an asymmetrical peak was obtained. The ascending part of the peak was found to mainly contain the cross-linked complex, while the descending one mainly corresponded to free lipase (fig. 4, insert). However, it must be pointed out that the partially purified cross-linked complex irreversibly lost approximately 80% of its initial specific activity during this purification, including dialysis, lyophilization and gel-filtration steps.

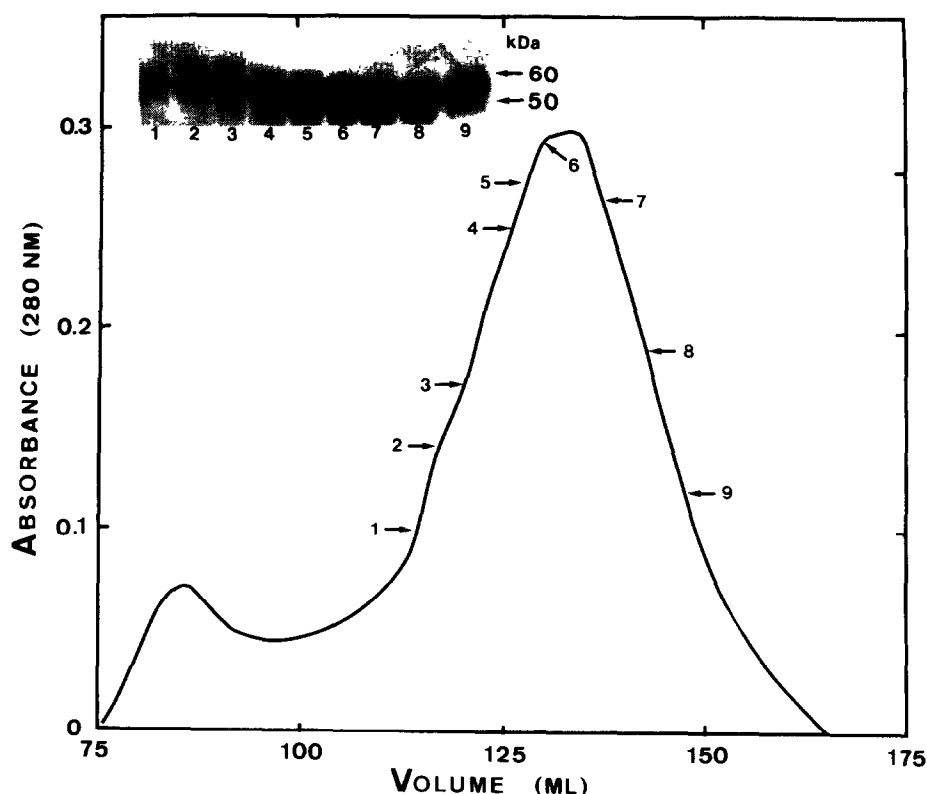


Fig. 4. Chromatography of the incubation medium on AcA 54 column. Lipase ( $2 \times 10^{-5}$  M) and colipase ( $1 \times 10^{-4}$  M) were incubated with CME-CDI (48 mM) for 10 min at 25°C as described in section 2 leading to about 40–50% of cross-linking. The reaction was stopped and the medium concentrated as described in the text. The concentrated medium was then loaded onto an AcA 54 column ( $0.9 \times 250$  cm) equilibrated with 10 mM Tris-HCl, pH 8.0 buffer containing 0.2 M NaCl and eluted with the same buffer. Flow rate, 25 ml/h; fraction volume, 2.5 ml. The numbered fractions indicated by arrows were analyzed by SDS polyacrylamide electrophoresis (insert).

#### 4. DISCUSSION

Among the several cross-linking reagents generally used, carbodiimides seem to be the best for studying protein associations involving ion pairing, as postulated for lipase-colipase. The main advantage of carbodiimides is to promote direct covalent cross-linking between the side chains involved in ion pairing without introducing a foreign spacer arm. This type of approach, i.e. stabilization of protein complexes by converting intermolecular ion pairing into covalent linkage, would bring us some structural information on the binding site of the proteins.

The cross-linking reaction between lipase and colipase, as described therein, led to the formation of a stoichiometric covalent binary complex. This finding further supports the assumption of ion pairing involved in the binding between the two partners [11]. Furthermore, an appropriate binding is likely to occur, since no loss of lipase and colipase activities on emulsified substrates was detected during the reaction. This finding is in agreement with the specific binding of the two proteins in the complex. The specificity of the lipase-colipase cross-linking reaction was emphasized by control experiments.

These cross-linking experiments show that, even in solution, lipase can recognize colipase. The lipase-colipase dissociation constant in solution was estimated at  $10^{-6}$  M [19] but it is several orders of magnitude higher than that estimated in the presence of a lipid interface ( $10^{-11}$  M) [3]. This suggests that the interfacial binding of colipase is expected to induce conformational changes leading to a higher affinity between lipase and colipase. The covalent binding did not affect the lipase catalytic activity, meaning that a correct binding between lipase and colipase occurs even in solution and that the lipase binding site for colipase can be assumed to be topologically different from the lipase catalytic site.

The stability of the isolated cross-linked complex was relatively low as compared to that of free lipase or colipase. Preliminary attempts to isolate the covalent complex led to a relatively high loss of activity, since only 20% of activity was retained in the purified com-

plex. Work is in progress to isolate the fully active cross-linked complex in order to investigate the catalytic properties of the lipase-colipase covalent complex as compared to those of the non-covalent system. Identification of the cross-linked side chains on lipase and colipase is also under investigation.

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