The Affinities of Procolipase and Colipase for Interfaces Are Regulated by Lipids

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ABSTRACT It has been suggested that at physiological pH, the trypsin-catalyzed activation of the lipase cofactor, procolipase, to colipase has no consequence for intestinal lipolysis and serves primarily to release the N-terminal pentapeptide, enterostatin, a satiety factor (Larsson, A., and C. Erlanson-Albertsson. 1991. The effect of pancreatic procolipase and colipase on pancreatic lipase activation. *Biochim. Biophys. Acta* 1083:283–288). This hypothesis was tested by measuring the adsorption of [^{14}C]colipase to monolayers of 1-stearoyl-2-oleoyl-*sn*-3-glycerophosphocholine and 13,16-*cis, cis*-docosadienoic acid in the presence and absence of procolipase. With saturating [^{14}C]colipase in the subphase, the surface excess of [^{14}C]colipase is 29% higher than that of procolipase, indicating that colipase packs more tightly in the interface. With [^{14}C]colipase-procolipase mixtures, the proteins compete equally for occupancy of the argon-buffer interface. However, if a monolayer of either or both lipids is present, [^{14}C]colipase dominates the adsorption process, even if bile salt is present in the subphase. If [^{14}C]colipase and procolipase are premixed for >12 h at pH ~8, this dominance is partial. If they are not premixed, procolipase is essentially excluded from the interface, even if procolipase is added before [^{14}C]colipase. These results suggest that the tryptic cleavage of the N-terminal pentapeptide of procolipase may be of physiological consequence in the intestine.

INTRODUCTION

In the intestine, dietary triacylglycerols and partial glycerides generated by the action of gastric lipase are hydrolyzed to fatty acids and 2-monoacylglycerols. The pancreatic enzymes responsible for this hydrolysis are triacylglycerol lipase (Verger, 1984) and carboxylester lipase (Rudd and Brockman, 1984), which are believed to function in a sequential fashion (Staggers et al., 1990). As a consequence of bile secretion and ingestion of phospholipids, the di and triacylglycerols on which these lipases act are present initially in emulsions stabilized by phospholipids and fatty acids (Carey et al., 1983). However, in model emulsion and monolayer systems, lipolysis is severely inhibited by the presence of phosphatidylcholine in the interface. This inhibition occurs for several reasons, the most important of which is the inability of the lipases to be adsorbed to bile salt- and phospholipid-rich interfaces (Borgström and Erlanson, 1971). Because the substrate on which the lipase acts is located in the interfacial phase, its hydrolysis is impeded or prevented.

Pancreatic triacylglycerol lipase, which first attacks the emulsified triacylglycerol in the intestine, is able to initiate substrate hydrolysis because the pancreas also secretes a cofactor protein, procolipase (Borgström and Erlanson, 1971). In contrast to lipase, procolipase and its trypsin-activated form, colipase, are better able to adsorb to phospholipid-rich interfaces. Both procolipase and colipase also form high affinity, 1:1 complexes with lipase and, hence, they provide a binding site for lipase at the lipid-water interface, especially when bile salts are present in the system (Borgström and Erlanson-Albertsson, 1984). Recently, the crystal structure of the lipase-procolipase B complex in the presence of bile salts and phospholipid has suggested a second role for the cofactor, participation in the interfacial activation of lipase (van Tilbeurgh et al., 1993). These results were confirmed using another complex containing lipase, octylglucoside, and colipase B (Egloff et al., 1995). The studies show the presence of a helical lid which, when open, exposes the recessed active site and an annulus of hydrophobic residues that strengthen interfacial binding. In the crystals, procolipase B and colipase B stabilize the lid in its open conformation. Additional roles for procolipase and colipase are suggested by the preferential interaction of procolipase with substrate, rather than phosphatidylcholine, in model lipid-water interfaces (Momsen et al., 1995). This preference may help target colipase to substrate-containing emulsion particles and should increase the two-dimensional concentration of substrate in the vicinity of the lipase-colipase complex.

Because procolipase and colipase share many functional properties, as measured in vitro, the significance of the proteolytic conversion of one form to the other has been debated. Early studies (Borgström et al., 1979; Wieloch et al., 1981), as well as more recent data (Rugani et al., 1995), indicated that colipase has a higher affinity for interfaces

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than procolipase. However, those data were obtained at nonphysiological pH values of 8.0–9.0. Other studies at a more physiological pH showed no difference in the abilities of procolipase and colipase to activate lipase in a model system (Larsson and Erlanson-Albertsson, 1991). Based on this observation it was suggested that the only functional consequence of the activation of procolipase by trypsin was to release the N-terminal pentapeptide, enterostatin, which may function as a satiety factor. Further contributing to the confusion in reported results is the recent report that the procolipase and colipase used in some of the work described above was probably proteolytically cleaved at the Ile^{79}-Thr^{80} bond during its purification (Rugani et al., 1995). The work reported herein addresses directly the question of whether the tryptic activation of procolipase to colipase regulates its interaction with simple interfaces through their competition for interfacial adsorption. The data obtained reveal that the ability of procolipase and colipase to compete for interfacial occupancy depends on the nature of the interface to which they are exposed, and suggest that the tryptic activation of procolipase to colipase may have functional consequences for intestinal lipolysis.

MATERIALS AND METHODS

1,3-Dioleoylglycerol (DO)[1] and 13,16-*cis*,*cis*-docosadienoic acid (DA) were from NuChek Prep, Inc. (Elysian, MN), 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) was from Avanti Polar Lipids (Alabaster, AL); tributyroylglycerol was from Aldrich (Milwaukee, WI); trypsin immobilized on polyacrylamide beads (cat # T8386) was from Sigma (St. Louis, MO); and sodium taurodeoxycholate was from Calbiochem (La Jolla, CA). For monolayer experiments sodium taurodeoxycholate was recrystallized three times from ethanol/water/diethyl ether (90:10:25) and then lyophilized from water. Solvents, water, buffer, and lipid solutions were prepared as described (Smaby and Brockman, 1990).

Colipase and procolipase were assayed by measuring their ability to stimulate the hydrolysis of tributyroylglycerol by pancreatic lipase in the presence of bile salts, as described previously (Momsen et al., 1995). One unit of activity corresponds to the release of butyric acid at the rate of 1 μmol/min at 24°C.

The isolation of the procolipase has been described previously (Momsen et al., 1995). In addition, some of the sample was dialyzed and concentrated by ultrafiltration in a 50-ml stirred cell fitted with a YM 3 membrane, (Amicon, Beverly, MA) to change buffer composition and pH. The specific activity of procolipase was 36,000 units/mg. The procedure for activation of procolipase to colipase was adapted from a published procedure (Rathelot et al., 1981). Immobilized trypsin, 76 units, 410 mg, was equilibrated 3 h with 40 ml 20 mM Tris, pH 8, at 4°C. The beads were pelleted by low-speed centrifugation, decanted, and resuspended in 40 ml buffer for 5 min. This procedure was repeated twice more to remove free trypsin. Procolipase, 125 mg in 20 mM Tris, ~7 mM NaCl, was added to the washed beads and the suspension stirred for 2.5 h at ~25°C. The extent of trypsin activation was monitored by ion exchange chromatography on Mono Q (Pharmacia, Piscataway, NJ). When activation was complete the beads were removed by filtration through Millex GV syringe filters (Millipore, Bedford, MA). The filtered colipase solution was purified by chromatography on Hiload Q (Pharmacia, Piscataway, NJ). Approximately 20 mg protein in 20 mM Tris, pH 8, was loaded on the column and eluted with a 500-ml linear 0.0–0.08 M NaCl gradient. Fractions containing colipase activity were concentrated and dialyzed against 10 mM potassium phosphate, pH 6.6, 0.1 M NaCl by ultrafiltration as above. The specific activity of the purified colipase was 41,000 units/mg.

Laser desorption time-of-flight mass spectrometry was performed on a Bruker Biflex (Bruker Instruments, Billerica, MA) operated in reflection mode. Colipase, 0.6 mg/ml in water, was diluted 1:1 with a solution of acetonitrile/water/trifluoroacetic acid (33:67:0.1 v/v/v). The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in acetone/trifluoroacetic acid (99.9:0.1 v/v). Sample solution and matrix solution, 1 μl each, were dried on a stainless steel target and introduced into the ion source of the instrument. Spectra were recorded by matrix-assisted laser desorption processes using a nitrogen laser of 337 nm. The N-terminal sequence (obtained through the University of Minnesota MicroChemical Facility) was shown to be glycine-isoleucine-isoleucine-isoleucine-asparagine, confirming the absence of the N-terminal pentapeptide of procolipase.

Reductive methylation of colipase was accomplished following published procedures (Jentoft and Dearborn, 1983). Purified colipase (53.6 mg in 45 ml 50 mM potassium phosphate, pH 6.6) was mixed with 2.5 ml freshly prepared 0.103 M $NaBH_3CN$. After 2 min, 2.5 ml [^{14}C]-HCHO in buffer (83 μCi, 33 mCi/mmol) was added and the mixture stirred for 2.5 h. The solution was concentrated by ultrafiltration as above to ~8 ml, and diluted with buffer. This was repeated five times for an overall diafiltration factor of ~2000. The radiolabeled colipase had a specific activity of 39,000 units/mg and a specific radioactivity of 9.76 μCi/μmol, corresponding to a labeling efficiency of ~30%. Procolipase, radiolabeled by the same procedure, had a specific activity of 41,080 units/mg and a specific radioactivity of 9.68 μCi/μmole.

The procedure followed for measuring adsorption of colipase to lipid monolayers is similar to that described for procolipase (Momsen et al., 1995), except that a single compartment trough, volume ~20 ml and area 20.3 cm^2, was used. As before, the subphase buffer was 10 mM phosphate, pH 6.6, 0.1 M NaCl and 0.01% NaN_3 at 24°C. Lipid solutions were calibrated so that careful addition of measured aliquots would give the desired surface pressure or surface concentration without monolayer compression. The lipid monolayer was allowed to stabilize for 5 to 10 min, after which [^{14}C]colipase, and in some cases procolipase, were added to the subphase. Surface pressure was monitored, typically for 30 min, and then the monolayer was collected on a solvent-cleaned, water-equilibrated hydrophobic paper. The paper was allowed to air-dry briefly, cut into ~1-cm square pieces, and mixed with 1 ml buffer and 10 ml scintillation cocktail for measurement of radioactivity (model LS 3801 liquid scintillation counter, Beckman Instruments, Fullerton, CA). As before, the values of adsorbed protein are corrected for carryover of nonadsorbed protein through the use of [^{32}P]phosphate in the subphase and for recovery of the lipid monolayer.

Gel permeation chromatography was performed using a Superose 12 H 10/30 FPLC column (Pharmacia, Piscataway, NJ). The column was equilibrated with 20 mM Tris, pH 8.0, 0.1 M NaCl at 0.4 ml/min. Mixtures of [^{14}C]colipase and procolipase were incubated at 4°C and pH 8 overnight. Protein was monitored by absorbance at 280 nm. Fractions, 0.5 ml, were collected for measurement of radioactivity, 80–85% of which was recovered. In one case, fractions containing protein and radioactivity were combined and used to measure [^{14}C]colipase adsorption to a DA monolayer for comparison to its behavior before the chromatography.

RESULTS

Colipase was generated by exposure of procolipase to immobilized trypsin following established procedures. This treatment was sufficient to remove the N-terminal pentapeptide, enterostatin, and a C-terminal tripeptide of no known functional significance. Amino acid analysis of the product was consistent with its being the 87 residue form of colipase, and this was confirmed by time-of-flight mass spectrometry (Fig. 1), which showed a predominant species with a mol wt of 9447.1. This value is in good agreement with the calculated mol wt of 9446.0 for the putative sequence

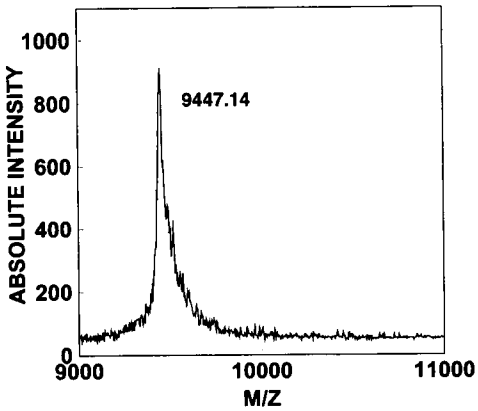

FIGURE 1 Laser desorption-time of flight mass spectrum of colipase. The value of 9447.14 shown is the molecular weight of the measurement of highest intensity. See Methods for experimental conditions. Sample size was 0.3 μg.

based on the corrected sequence of (pro)colipase recently published (Rugani et al., 1995).

Initial attempts to quantitate the adsorption of this colipase to argon- and lipid-water interfaces were disappointing in that the anticipated extents of coverage, based on earlier measurements with procolipase (Momsen et al., 1995), could not be obtained. Subsequent investigation established that the unexpectedly low adsorption of colipase resulted from a methodological problem in its analysis. Specifically, it was found that the hydrophobic paper, onto which the argon- or lipid-water interface containing colipase was collected, retained significant quantities of colipase after being eluted with detergent solution. Because procolipase was quantitatively eluted under the same conditions, this observation showed qualitatively that there was a difference in the affinities of procolipase and colipase for the hydrophobic paper, and suggested that the proteolytic conversion of procolipase to colipase might have functional consequences.

To avoid uncertainties associated with the elution of colipase from the hydrophobic paper, a procedure was developed in which the colipase was left on the paper and quantitated radiometrically. This procedure also eliminated the intrinsic error of $\pm 5\%$ of the titrametric assay procedure. As described in Materials and Methods, colipase was radiolabeled by reductive methylation following established procedures. The resulting product had an average of 0.3 [^{14}C]-methyl groups per colipase, presumably attached to lysine. It was shown earlier that 240 nM procolipase was sufficient to saturate either the argon- or lipid-buffer interface with protein (Momsen et al., 1995). This was confirmed for [^{14}C]colipase at 235 nM. With this concentration added to the aqueous phase, the surface excess of [^{14}C]colipase at the argon-buffer interface was 30.1 $\pm$ 1.1 pmol/cm^2 (n = 9). The accompanying surface pressure change induced by colipase addition to the subphase, 18.4 $\pm$ 1.9 mN/m, was similar to the value of 17.7 $\pm$ 0.4 mN/m obtained earlier using procolipase. However, the value of 23.3 $\pm$ 3 pmol/cm^2 determined for the surface excess of procolipase under comparable conditions (Momsen et al., 1995), but using an enzymatic procedure for procolipase determination, is significantly lower than measured using [^{14}C]colipase. This suggested either a tighter packing density for [^{14}C]colipase or a methodological difference. To test this latter possibility, a small quantity of radiolabeled procolipase was synthesized following the same protocol used to make [^{14}C]colipase. It adsorbed to 24.4 $\pm$ 1.6 pmol/cm^2 (n = 3), supporting the notion that [^{14}C]colipase adsorbs to the argon-buffer interface in a more compact conformation than procolipase.

The difference in the behavior of [^{14}C]colipase compared to procolipase could be the result of [^{14}C]colipase having a higher surface activity relative to native colipase or of colipase and procolipase having different surface activities. To address the first possibility, mixtures of [^{14}C]colipase and colipase were prepared and injected under a clean argon-buffer interface and the surface excess of [^{14}C]colipase determined. As shown in Fig. 2, filled circles, there is a proportional relationship between the percentage of [^{14}C]colipase added to the subphase and the surface excess of [^{14}C]colipase recovered from the interface. The lines shown in Fig. 2 are theoretical lines drawn between zero and the average of values obtained using [^{14}C]colipase alone. The most straightforward explanation for this proportionality is that [^{14}C]colipase and native colipase compete equally

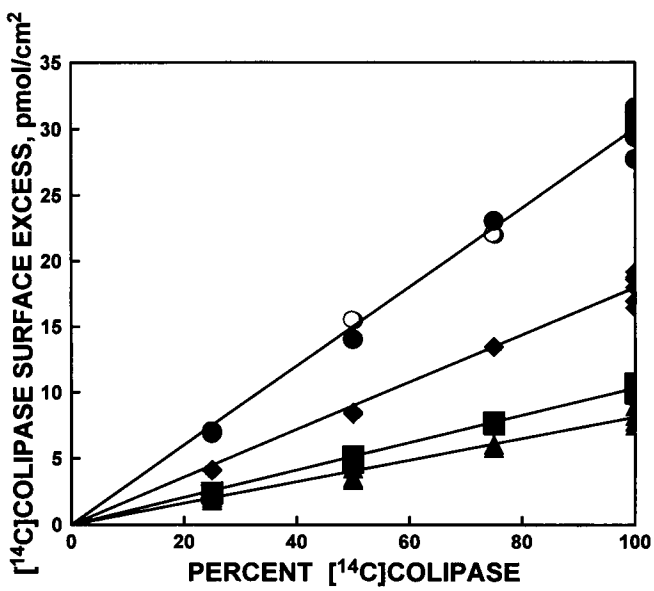

FIGURE 2 Dependence of the surface excess of [^{14}C]colipase on its abundance in the aqueous phase for mixtures of [^{14}C]colipase and native colipase. Proteins were mixed for 2 min (*open symbols*) or overnight (*closed symbols*) before addition to the subphase. After 30 min the amount of [^{14}C]colipase was measured at the argon-buffer interface (●, ○) or under lipid monolayers of DA (◆), SOPC (▲), or DA/SOPC mol fraction 0.5 (■). The initial pressure of the lipid monolayer was 2 to 4 mN/m. Total concentration of [^{14}C]colipase plus native colipase in the subphase was 235 nM. The theoretical solid line for each data set is drawn between 0 and the average of the values obtained at 100% [^{14}C]colipase.

well for the argon-buffer interface. Because it had been shown earlier that procolipase exhibits preferential interactions with different lipid classes (Momsen et al., 1995), lipid-protein interactions could differentially affect the interactions of colipase and [^{14}C]colipase with the interface. To test this possibility, comparisons were performed as above except that a lipid monolayer was initially present at the interface. The lipids used were SOPC, DA, or their equimolar mixture. Based on data obtained with procolipase, the surface concentration of lipid used in each case was that expected to occupy about half the interface in the presence of saturating [^{14}C]colipase. As shown in Fig. 2, excellent proportionality between [^{14}C]colipase added and adsorbed was obtained with each type of lipid monolayer. Overall, the results in Fig. 2 show that the derivatization of native colipase by reductive methylation results in a radiolabeled species which behaves, both in the presence and absence of lipids, like the unlabeled colipase from which it was generated. Thus, the difference in surface activity between [^{14}C]colipase and procolipase at the argon-buffer interface noted above is not due to the radiolabeling procedure.

Next addressed was the ability of procolipase to compete with [^{14}C]colipase for surface occupancy. The protocol was identical to that employed for [^{14}C]colipase/native colipase competition, in that [^{14}C]colipase and procolipase were premixed in concentrated solution and incubated for at least 12 h before aliquots were injected into the aqueous phase. Except where noted below, the total concentration of [^{14}C]colipase and procolipase added to the aqueous phase was 235 nM. As shown in Fig. 3 *A*, filled circles, [^{14}C]colipase and procolipase compete equally well for surface occupancy at the argon-buffer interface. However, in the presence of a lipid monolayer, the result is markedly different. As shown in Fig. 3, *B–D*, filled circles, lowering the proportion of [^{14}C]colipase in the subphase did not produce a proportional drop in the level of [^{14}C]colipase adsorbed. In particular for SOPC, at a subphase abundance of only 10% of [^{14}C]colipase + procolipase, [^{14}C]colipase was present at the interface at more than 50% of the value expected if only [^{14}C]colipase had been added to the subphase. With DA that was true when 25% [^{14}C]colipase was added to the subphase, but the value fell more toward the expected proportionality at 10% [^{14}C]colipase in the mixture. Calculations showed that the total amount of [^{14}C]colipase in the subphase at 10% of the mixture was of the same magnitude as the amount of [^{14}C]colipase adsorbed to the DA monolayer plus what might adsorb to the walls of the Teflon trough. Thus, it was possible that there was insufficient [^{14}C]colipase in the subphase to saturate the interface with DA at low [^{14}C]colipase compositions. To test this possibility, experiments with DA monolayers were repeated using twice the total [^{14}C]colipase + procolipase. The results, shown in Fig. 3 *B*, open squares, indicate that with twice the total [^{14}C]colipase + procolipase, the measured surface excess of [^{14}C]colipase when it constitutes 10% of that added to the subphase is again over half of that expected if only [^{14}C]colipase were present in the subphase.

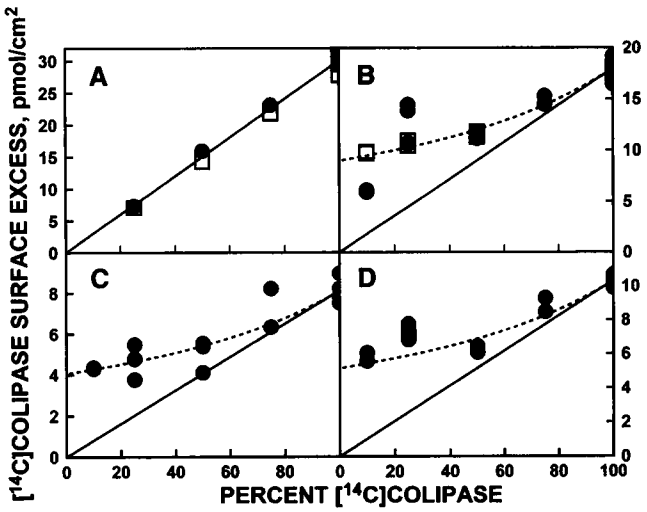

FIGURE 3 Dependence of the surface excess of [^{14}C]colipase on its abundance in the aqueous phase for mixtures of [^{14}C]colipase and native procolipase. Panel *A*, argon-buffer interface premixing time of 2 min (□) or overnight (●). Panels *B–D*, cofactors incubated overnight before injection under a lipid monolayer at 2–4 mN/m of DA (*B*), SOPC (*C*), or their equimolar mixture (*D*). Total [^{14}C]colipase plus native procolipase in the aqueous phase, 235 nM except for panel *B* open symbols, 470 nM. The theoretical solid line in each panel is drawn between 0 and the average of the surface excess values obtained at 100% [^{14}C]colipase. The dashed line is the surface excess of [^{14}C]colipase predicted by the dimer model (see text).

This is comparable to what was observed with SOPC, where total [^{14}C]colipase adsorption is lower. These results suggest that whatever species of cofactor protein is causing the behavior exhibited in Fig. 3, *B–D* becomes limiting at sufficiently low [^{14}C]colipase + procolipase.

Consideration of the behavior observed with lipid monolayers and excess [^{14}C]colipase + procolipase, Fig. 3, *B–D*, suggested the possibility that [^{14}C]colipase and procolipase might form random homo- and/or hetero-dimers or higher aggregates. Assuming that any dimer species containing [^{14}C]colipase adsorbs to lipid monolayers in preference to procolipase-procolipase dimers, a simple statistical model can be derived for predicting the surface excess of [^{14}C]colipase. The calculated values are shown in Fig. 3, *B–D* as dotted lines, which are normalized to the adsorption measured when only [^{14}C]colipase was added to the subphase. Within the scatter of the data, agreement with the model is excellent. Based on this, experiments were carried out to determine if dimers were, indeed, present in the parent solutions or [^{14}C]colipase-procolipase mixtures. These included ultracentrifugation, quasielastic light scattering, and gel permeation chromatography. None of these methods revealed any measurable proportion of dimer or higher aggregate species in either of the component protein solutions or their mixtures, regardless of sample history. For example, gel permeation chromatography of the radiolabeled protein showed a single, sharp peak (Fig. 4), which had the molecular weight of monomeric colipase or procolipase (calibration not shown). Additionally, adsorption of

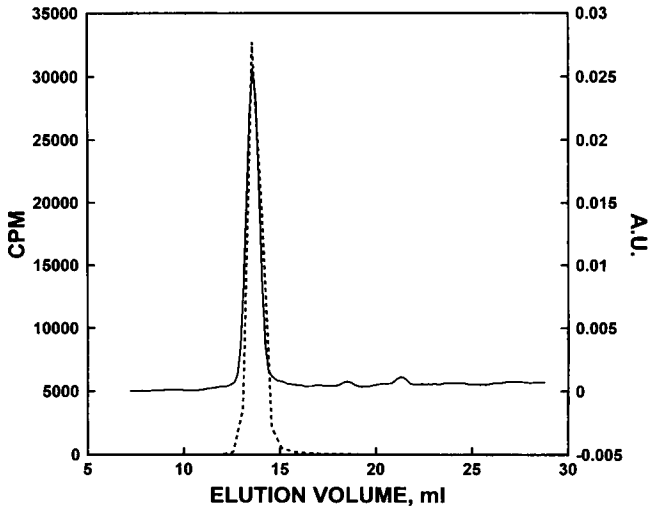

FIGURE 4 Gel permeation chromatography of a 1:1 mixture of [^{14}C]colipase and native procolipase. Sample, 9.6 nmol, preincubated overnight at 4°C, pH 8; A_{280} (—); radioactivity (- - -).

[^{14}C]colipase to a docosadienoic acid monolayer from a 1:3 premixed solution of [^{14}C]colipase and procolipase was the same before and after gel permeation chromatography of the mixture as illustrated in Fig. 4 (not shown). Thus, there is no direct evidence to support the dimer model in spite of the apparent agreement of the data, shown in Fig. 3, *B–D*, with it (see Discussion).

Regardless of the applicability of the dimer model, it appears that [^{14}C]colipase has a higher affinity for the lipid-buffer interface than procolipase when the surface is exposed to a premixed solution of both species. To determine if this was affected by the conditions of the preincubation, additional experiments were carried out. For these the [^{14}C]colipase and procolipase were premixed immediately before being injected into the aqueous phase, were injected simultaneously or were injected sequentially with a time interval between additions. These experiments were carried out with monolayers of DA, and in some cases with SOPC. The results, which are summarized in Table 1, showed a pattern of behavior that differs substantially from that shown in Fig. 3, *B–D*. The column labeled 'percent expected' gives the adsorption as a percent of what would be obtained if only [^{14}C]colipase had been injected into the subphase at the same total protein molarity. Simply, the data in Table 1 show that if [^{14}C]colipase and procolipase are not premixed 12 h or more before use, [^{14}C]colipase completely dominates the adsorption process, i.e., procolipase appears

TABLE 1 Dependence of [^{14}C]colipase adsorption to lipid monolayers on sample history and order of addition

Lipid	1st addition*,#	Time (min)§	2nd addition*	Time (min)§	Total cofactor (μM)	Final mol % ^{14}C	[^{14}C]colipase (pmol/cm^2)	Percent expected
DA	^{14}C	30			235	100	18.8	100
	^{14}C + P	20			235	50	22.1	118
	^{14}C + P	30			235	50	21.7	115
	^{14}C + P	60			235	50	18.3	97
	^{14}C + P	120			235	50	20.5	109
	^{14}C + P	995			235	50	22.5	120
	^{14}C,P	60			470	50	18.0	96
	P	15	^{14}C	15	235	50	20.8	111
	^{14}C	15	P	15	235	50	22.2	118
	^{14}C + P¶	5	^{14}C	30	470	100	16.8	89
	^{14}C + P¶	5	^{14}C + P	30	470	50	17.4	93
	[^{14}C + P]	15			235	50	12.9	69
	[^{14}C + P]	60			235	50	11.6	62
	[^{14}C + P]	120			235	50	11.3	60
	[^{14}C + P]	1050			235	50	13.4	71
	P	30	[^{14}C + P]	30	470	33	11.1	59
	C	30	[^{14}C + P]	30	470	33	2.8	45
	[^{14}C + P]	30	C	30	470	33	3.6	58
SOPC	^{14}C	30			235	100	8.6	100
	P	15	^{14}C	15	235	50	8.6	100
	^{14}C + P	30			235	50	8.2	95
	^{14}C + P¶	5	^{14}C	30	470	100	6.5	76
	^{14}C + P¶	5	^{14}C + P	30	470	50	5.4	63
	[^{14}C + P]	30			235	50	5.4	63
	[^{14}C + P]	30			235	25	3.8	44

*C, colipase; ^{14}C, [^{14}C]colipase; P, procolipase. If two additions, each contained same total of C + ^{14}C + P as indicated.
#C + P, mixed immediately before addition to subphase; C, P, added sequentially to subphase; [C + P], premixed overnight before addition to subphase.
§Subphase stirred at 50–100 rpm during incubation.
¶Subphase contained 0.1 mM taurodeoxycholate.

to be completely displaced from the interface by [^{14}C]colipase. This is true even if procolipase is added 15 min before [^{14}C]colipase, during which time the surface pressure increases (not shown). This increase is an indication of procolipase adsorption to the interface. Comparable control experiments were carried out in which the [^{14}C]colipase and procolipase were premixed overnight. In this case, adsorption of [^{14}C]colipase was 50–70% of that expected if [^{14}C]colipase adsorption was dominant (Table 1). This result is consistent with Fig. 3, *B–D*. Note also in one of these experiments carried out with DA monolayers that nonradiolabeled colipase, added before an equal quantity of premixed procolipase + [^{14}C]colipase, appeared to compete with [^{14}C]colipase for interfacial occupancy. Overall, the data in Table 1 show that the anomalous behavior shown in Fig. 3, *B–D* depends on overnight preincubation of the procolipase and [^{14}C]colipase. More importantly, however, the data show that in the absence of preincubation, [^{14}C]colipase quantitatively prevents procolipase adsorption at the interface or replaces it rapidly if procolipase was added before [^{14}C]colipase. This implies not only that [^{14}C]colipase has a higher affinity for the interface than procolipase, but also that the spontaneous dissociation rate of procolipase or its displacement rate by [^{14}C]colipase from the interface is relatively fast.

Prior work suggesting the equivalency of procolipase and colipase in their interaction with interfaces was performed in the presence of bile salt (Larsson and Erlanson-Albertsson, 1991). To determine if bile salt was responsible for the difference between our data and theirs, additional adsorption experiments were conducted with 100 μM taurodeoxycholate in the subphase. At this concentration the surface excess of the bile salt is near its maximum, but micelles are not present. Under these conditions the monolayer is stable over the time course of the experiment, even when a fatty acid is used. As shown in Table 1, for SOPC and DA monolayers the presence of bile salt reduced adsorption of [^{14}C]colipase alone to 89 and 76% of the values obtained in the absence of the bile salt. When an equimolar mixture of [^{14}C]colipase and procolipase was injected in the presence of bile salt, the values were 93 and 63% of expected, normalized to the absence of bile salt. If these latter values are corrected for adsorption of [^{14}C]colipase alone observed in the presence of the bile salt, the detergent has no effect on adsorption to DA monolayers and reduces relative adsorption to SOPC by only 27%. Thus, the preferential adsorption of [^{14}C]colipase is observed even in the presence of bile salt.

The anomalous effects of preincubation demonstrated with procolipase-[^{14}C]colipase mixtures necessitated reexamination of the results shown for [^{14}C]colipase+colipase and for [^{14}C]colipase+procolipase mixtures at the argon-buffer interface. As shown in Fig. 2, open circles, and Fig. 3 *A*, open squares, sample history had no effect on adsorption to the argon-buffer interface. These results confirm that the radiolabeling procedure did not affect the surface properties of colipase. Thus, the effect of preincubation, like the dominance of [^{14}C]colipase adsorption over that of procolipase, is evident only when lipid is present at the interface.

Examination of the preincubated mixtures containing [^{14}C]colipase and procolipase showed that their pH was ≈8 as a consequence of the pH and buffer strength of the procolipase stock solution used. To determine the importance of pH in eliciting the preincubation effect, two mixtures containing 25% [^{14}C]colipase and 75% procolipase were prepared. One was prepared from the stock solutions used in other experiments described above so that its pH was ≈8. A sample of this solution was withdrawn and immediately assayed for the adsorption of [^{14}C]colipase to a DA monolayer. After subsequent incubation for 17.5 h at 4°C its pH was lowered to 6.6 using 1 M potassium phosphate buffer, pH 6.4. Additional samples were then withdrawn as a function of time and assayed for the adsorption of [^{14}C]colipase to DA monolayers. The second sample was prepared from stock solutions adjusted to pH 6.6 before the [^{14}C]colipase and procolipase were mixed. Aliquots of this solution were also withdrawn immediately and at time intervals thereafter for assay of [^{14}C]colipase adsorption. Fig. 5 shows a logarithmic plot of the percent of the surface excess of [^{14}C]colipase adsorbed to the DA monolayer as a function of time for each sample. The upper line shows results obtained with the sample that was prepared at pH 6.6, and the lower shows those obtained after the high pH sample was incubated overnight and then adjusted to pH 6.6. The vertical difference between the intercepts of the two lines shows the extent of decrease in [^{14}C]colipase adsorption, about 25%, that occurred as a result of the incubation at high pH for 17.5 h. From this difference an estimate of ~37 h for the half life of this decrease can be calculated. The declining values of both samples with time after adjustment to pH 6.6 show that the unknown process

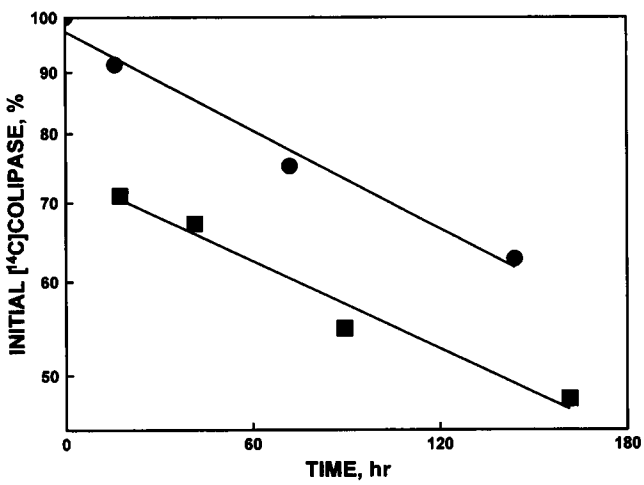

FIGURE 5 Dependence of [^{14}C]colipase adsorption to fatty acid monolayers on sample history. Mixtures containing 25% [^{14}C]colipase and 75% procolipase were prepared at pH 6.6 (●) or 8.0 with adjustment after 17.5 h to 6.6 (■) as described in the text and assayed with time for adsorption to DA monolayers at an initial surface pressure of about 3 mN/m. Total [^{14}C]colipase plus native colipase, 235 nM.

also occurs at more physiological pH. Assuming a simple first-order model, the half life for this process at pH 6.6, calculated from the average of the slopes of the fitted lines, is ~220 h.

One possible explanation for the data above and in Table 1 is that [^{14}C]colipase becomes modified at pH 8.0 in such a way as to decrease its surface activity. To test this possibility, a sample of [^{14}C]colipase stock solution was adjusted to pH 8.0 and incubated alone for 67 h at 4°C. This was then mixed with unlabeled colipase stock solution at pH 6.6 to yield mixtures containing 25, 50, and 75% [^{14}C]colipase. Each mixture was then injected to a final concentration of 235 nM below a monolayer of SOPC/DA (1:1) initially at a surface pressure of 3 mN/m, as used in the other studies. As controls, identical experiments were carried out using [^{14}C]colipase, which had not been incubated at pH 8.0. The measured surface excess values obtained with [^{14}C]colipase that had been preincubated at pH 8.0 were 87, 94, and 98% of the respective control values, indicating that the surface activity of [^{14}C]colipase is minimally affected by its incubation alone at high pH. This suggests that whatever is responsible for the time-dependent effect at pH 8.0, and to a lesser extent at pH 6.6, depends on the [^{14}C]colipase and procolipase being premixed.

DISCUSSION

Colipase was generated from the 95-residue form of procolipase by digestion with trypsin. Because cleavage of the N-terminal pentapeptide alone does not change the net charge of the molecule, conditions were selected that should cause a cleavage at residue Arg_{92} as well (Rathelot et al., 1981). This was done to facilitate separation of the product from the starting material. The structure of the expected 87-residue form of colipase (residues 6–92 relative to procolipase) obtained was verified by time-of-flight mass spectrometry (Fig. 1), which indicated the appropriate molecular weight as reported in a recent study (Rugani et al., 1995). At the C-terminal end of the molecule this species differs by only one residue from procolipase B (residues 1–93), which has been used to determine both the crystal structure (van Tilbeurgh et al., 1993) and the solution structure (Breg et al., 1995) of procolipase in a complex with lipase and detergent. Colipase B (residues 6–93 relative to procolipase) was recently used for determination of the crystal structure of a lipase-colipase B-detergent complex. (Egloff et al., 1995). That this one residue difference at the C-terminus is probably functionally unimportant is indicated by several observations. The solution structure of procolipase B showed very poorly defined structure for the C-terminal residues beyond 89. More directly, it was observed that in the ternary crystalline complexes containing procolipase B and colipase B no residue beyond 90, relative to the original procolipase structure, shows any electron density. On this basis it was concluded that the C-terminal part of the molecule does not bind either lipase or lipid (Egloff et al., 1995). Lastly, injection of colipase B under egg phosphatidylcholine monolayers at increasing initial surface pressures causes pressure changes that linearly extrapolated to zero at an initial pressure of 30 mN/m (Rugani et al., 1995). This is close to the extrapolated value of 27 mN/m obtained under similar conditions using monolayers of 1-stearoyl-2-oleoylphosphatidylcholine and the [^{14}C]colipase used in the present study (M. Momsen and H.L. Brockman, unpublished observations).

The method of reductive methylation, which has been used by others for radiolabeling of surface active proteins (e.g., (Weinberg et al., 1992)), was chosen to perturb the surface properties of colipase as little as possible and retain total electrical charge. The data in Fig. 2 show that the [^{14}C]colipase produced behaved in the same manner as colipase both in the presence and absence of lipids. This indicates that the maximal surface excess of 30.1 pmol/cm^2 in the absence of lipid being 29% higher than that reported earlier for procolipase (Momsen et al., 1995) is not due to the radiolabeling procedure. Also, we have recently radiolabeled the procolipase from which colipase was synthesized and shown that its surface excess is, within error, identical to that reported based on the catalytic assay. Thus, the difference in the surface excesses of procolipase and colipase at the argon-buffer interface cannot be ascribed to methodological differences. Rather, the data indicate strongly that colipase can pack far more tightly than procolipase at the interface. This difference is presumably regulated by the N-terminal pentapeptide of the molecule which, like the C-terminal residues beyond 90, were unstructured in solution (Breg et al., 1995) and in crystals of the lipase-procolipase B-detergent ternary complex (van Tilbeurgh et al., 1993). This suggests that the presence of the pentapeptide must interfere with the close packing of the procolipase molecules in the absence of lipid.

Procolipase and colipase each form a 1:1 complex with pancreatic lipase and anchor it to the lipid-water interface in the presence of bile salts. Early reports showed that colipase was much more efficient than procolipase in shortening the lag period observed in lipase-catalyzed hydrolysis of substrates presented to the enzyme as phospholipid-stabilized triacylglycerol emulsions (Borgström et al., 1979) or as diacylglycerol monolayers (Wieloch et al., 1981). On the basis of this it was concluded that the proteolytic activation of procolipase to colipase facilitated its adsorption and, hence, that of lipase, to phospholipid-covered surfaces. Later, fatty acid products of lipolysis were specifically identified as strengthening this interaction by forming clusters of their calcium salts. These experiments were performed at a pH $\geq$ 8 and in the presence of millimolar calcium. However, duodenal pH is typically $\leq$ 7 and calcium salts of fatty acids have been found to be a minor component when human duodenal contents were analyzed (Staggers et al., 1990). When the lag phenomenon was reinvestigated at pH $\leq$ 7.0 (Larsson and Erlanson-Albertsson, 1991) little or no difference in the lag phase or steady-state hydrolysis rate of phospholipid-stabilized triacylglyc

erol emulsions was observed when procolipase was replaced by colipase. The apparent K_m's for the hydrolytic reaction determined with procolipase and colipase were also nearly identical. Additionally, there is extensive evidence that enterostatin, a tryptic pentapeptide released when procolipase is converted to colipase, functions as a satiety factor (Erlanson-Albertsson, 1992). The combination of these results led (Larsson and Erlanson-Albertsson, 1991) to the conclusion that at physiological pH, the proteolytic cleavage of procolipase may have little effect on lipid digestion and serve primarily to release enterostatin.

The results of the competition adsorption experiments involving procolipase and [^{14}C]colipase at the argon-buffer interface (Fig. 3 *A*) are consistent with the proteins having equivalent surface activity. This equivalence was observed irrespective of the history of the sample before being injected into the aqueous subphase beneath the argon-buffer interface. However, the comparative adsorption experiments conducted with a lipid monolayer initially present at the interface clearly challenge the notion that the tryptic activation of procolipase to colipase is of no direct consequence for duodenal lipolysis. At pH 6.6 the presence of [^{14}C]colipase in mixtures with procolipase led to its being adsorbed to lipid monolayers in preference to procolipase when both were added simultaneously, and to the quantitative displacement of procolipase when the proteins were added sequentially (Table 1). As shown in Figs. 3 *B–D* and Table 1, whether the lipid was fatty acid or phospholipid made no difference in the behavior, beyond controlling the total level of [^{14}C]colipase adsorption. This was somewhat unexpected because an earlier study of procolipase adsorption to lipid monolayers showed marked differences between its affinity for phosphatidylcholine versus fatty acid or diacylglycerol (Momsen et al., 1995). In the absence of preincubation at high pH, the dominance of [^{14}C]colipase is complete (Table 1). From this it can be inferred that the N-terminal pentapeptide of procolipase limits interaction with the lipid chains. These differences in lipid packing with protein were not observed in the crystal structures of the lipase-detergent-procolipase B and -colipase B complexes (van Tilbeurgh et al., 1993; Egloff et al., 1995), but an octylglucoside molecule was observed to interact with the N-terminal region in the colipase B-containing complex.

The ability of procolipase to compete for interfacial occupancy in the presence of lipid was found to increase upon incubation of procolipase and [^{14}C]colipase at high pH (Fig. 3, *B–D* and Table 1). Moreover, this increase depended upon the two forms of the cofactor being incubated together. These observations eliminate explanations based on procolipase-[^{14}C]colipase interactions occurring in the lipid-water interface. For samples mixed for 12 h or more at high pH, the extent of [^{14}C]colipase adsorption measured as a function of its abundance in the mixture suggested the formation of dimers or higher protein aggregates. A likely mechanism for such dimerization would be disulfide interchange among the five disulfide cross-links in both species. However, no direct evidence for dimers or higher aggregates has been found, and a preincubated sample of procolipase-[^{14}C]colipase exhibited the same extent of [^{14}C]colipase adsorption before and after gel permeation chromatography.

The lack of demonstrable dimer formation and the lack of a pH dependence of the surface activity of [^{14}C]colipase suggest that procolipase is being modified during the preincubation at high pH in the presence of [^{14}C]colipase. This could be the result of traces of trypsin in the colipase, which would slowly clip the labile N-terminal peptide from procolipase, converting it to colipase 89 (6–95 relative to procolipase). However, the trypsin used to activate the procolipase was immobilized and was extensively washed before use. In addition, the product colipase was purified by ion exchange chromatography. An alternative hypothesis is that colipase itself might be able to slowly activate procolipase. It has been reported that procolipase, colipase, and apolipoprotein CII have the ability to hydrolyze 4-methylumbelliferyloleate in a manner that is inhibited by phenylmethylsulfonylfluoride. On this basis it was proposed that these cofactors may function as acyl-enzyme hydrolases for their respective lipases (Vainio et al., 1980). Although this physiological role was later discounted (Balasubramaniam et al., 1986), it is possible that colipase exhibits enough nonspecific hydrolytic activity to cleave the N-terminal pentapeptide of procolipase when they are mixed together for long periods in high concentration at high pH. Whatever the mechanism by which procolipase becomes altered under these experimental conditions, its time course at physiological pH (Fig. 5) makes it unlikely that such conversion is physiologically relevant. Thus, colipase adsorption should dominate in colipase-procolipase mixtures in vivo. If so, our results suggest that the activation of procolipase to colipase in the intestine may have direct consequences for its ability to serve as a cofactor for the lipase-catalyzed hydrolysis of glycerides.

Comparison of our results with others (Larsson and Erlanson-Albertsson, 1991) suggests several possible reasons for the difference in results between studies. One is that, for technical reasons, our system did not include micellar concentrations of bile salts in the aqueous phase. However, a concentration sufficient to saturate the surface with bile salt did not eliminate the dominant adsorption of [^{14}C]colipase (Table 1). Additionally, both procolipase and colipase enable lipase adsorption to the cofactor in the presence of bile salts and triacylglycerol (Borgström and Erlanson-Albertsson, 1984). The other possibility is the surface-to-volume ratio in the systems. The amount of Intralipid used in the earlier study was not specified, but it is possible that it was sufficient to saturate (pro)colipase adsorption as well as that of colipase. This might have masked any differences in the relative affinities of the proteins for the interface. Also, the present study used saturating quantities of cofactor rather than catalytic amounts. Experiments to resolve these differences will provide the basis of future studies. In any event, the present results show that colipase will quantitatively prevent or displace procolipase from model lipid-buffer

interfaces when the proteins compete directly for interfacial occupancy.

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