How Colipase−Fatty Acid Interactions Mediate Adsorption of Pancreatic Lipase to Interfaces†

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Received December 8, 1997; Revised Manuscript Received March 20, 1998

ABSTRACT: Colipase is a cofactor protein which forms a 1:1 complex with pancreatic lipase. This facilitates lipase adsorption to phosphatidylcholine-rich interfaces, presumably as a consequence of the higher affinity of colipase for such interfaces. According to this model, the presence of colipase in an interface should be sufficient to enable lipase adsorption from the aqueous phase. To test this hypothesis, mixed monolayers of colipase, phosphatidylcholine, and fatty acid at the argon−buffer interface were exposed to lipase injected into the stirred aqueous subphase. Spread colipase remained associated with the lipid monolayer in a surface pressure- and lipid composition-dependent manner. For example, with diacylphosphatidylcholine alone, colipase remained in the lipid monolayer at surface pressures ≤20 mN/m, but with pure fatty acid this was increased to ∼40 mN/m. Contrary to the existing paradigm, the presence of colipase in a lipid monolayer was not sufficient to enable the adsorption of lipase to the interface. Fatty acid was also required, and its ability to enhance lipase adsorption over that observed in the absence of colipase was dependent on the fatty acid and colipase mole fractions. These results support the hypothesis that colipase concentrates fatty acids laterally at its periphery and suggest that, together with lipase−colipase interaction, the fatty acid-rich nano-domain surrounding colipase facilitates lipase adsorption in the 'flap-opened' conformation.

Following ingestion of a triacylglycerol-rich meal, partial lipolysis of dietary triacylglycerol occurs in the stomach, catalyzed by gastric or lingual lipases (*1*, *2*). This together with mechanical forces in the stomach generates an emulsion of triacylglycerols, diacylglycerols, and fatty acids which passes into the intestinal lumen (*3*, *4*). In the lumen, bile salts and phospholipids, particularly diacylphosphatidylcholine, are added to stabilize the emulsion particles (*5*). The diacyl- and triacylglycerols in these particles are then attacked by pancreatic and carboxylester lipases to complete triglyceride digestion. There is evidence that pancreatic triacylglycerol lipase (PTL),[1] a 50 kDa protein, is the primary agent which attacks the emulsion particles and that carboxylester lipase functions secondarily on micellar or other phases (*6*, *7*). These phases appear as lipolytic products that accumulate faster than they are removed through the intestinal wall (*8*−*10*).

Recent crystallographic studies of lipases in general have shown that most possess a "lid" or "flap" which covers the deeply recessed active site. This lid is presumably closed when the enzymes are in solution but assumes an open conformation in the presence of a lipid−water interface (*11*). This change in conformation, which accompanies lipase adsorption to interfaces, involves the exposure of both the active site and hydrophobic residues which line the lid. This exposure of the active site is believed to be responsible for the much higher activities of lipases in interfaces as opposed to solution, i.e., interfacial activation.

In vitro, bio-surfactants such as phospholipids (*12*, *13*), bile salts (*14*−*16*), or both (*17*, *18*) inhibit PTL activity. The water-insoluble lipid substrates of PTL reside in the particle surface and bulk phases, and their hydrolysis can occur only if the enzyme also resides in the lipid−water interface (*7*). Phospholipids and bile salts prevent PTL adsorption to the emulsion surface (*19*) and, hence, are inhibitory. In vitro, and in vivo, this inhibition is overcome by the presence of colipase, a 9.4 kDa cofactor protein (*1*, *20*−*23*). Colipase is synthesized as a procofactor in the pancreas, secreted, and activated by trypsinolysis of an N-terminal pentapeptide to its mature form (*22*, *24*). Early studies suggested that the ability of colipase to enable lipolysis in the presence of surfactants was a consequence of (a) its ability to form a 1:1 complex with lipase both in solution (*17*, *25*, *26*) and at the lipid−water interface (*17*) and (b) its higher affinity for surfactant-rich interfaces (*5*, *14*, *15*). Thus, the primary role of colipase is to anchor lipase to the substrate-containing interface in the presence of bile salts and phospholipids. Biochemical (*27*), molecular biological (*28*), and crystallographic (*29*) studies have determined that this anchoring involves protein−protein contacts between colipase and the C-terminal domain of PTL. In addition, the PTL lid, located in the N-terminal domain, has contacts with colipase which help to stabilize the enzyme in the lid-open conformation (*30*, *31*). Hence, the interfacial anchoring function of colipase includes stabilization of the enzyme in the open conformation to expose its active site.

† Supported by USPHS Grant HL49180 and the Hormel Foundation.

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[1] Abbreviations: PTL, porcine, colipase-dependent, pancreatic triacylglycerol lipase (E.C. 3.1.1.3); DA, 13,16-*cis*,*cis*-docosadienoic acid; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

S0006-2960(97)03015-8 CCC: $15.00
Published on Web 05/19/1998

The overall anchoring function of colipase can qualitatively explain its biological function and its ability to shorten time delays preceding hydrolysis of emulsified substrates in vitro. However, there are indications in the literature of an additional role of colipase involving free fatty acid. In vivo, fatty acids are generated by the partial hydrolysis of triacylglycerols in the stomach (*32*). At pH ≥8.0 and in the presence of Ca^{2+} at low concentrations, the colipase-dependent, PTL-catalyzed hydrolysis of glycerides is facilitated by the presence of fatty acids and by activation of procolipase to colipase (*8*, *12*, *22*, *33*, *34*). Moreover, it has been shown that the clustering of Ca^{2+} soaps of fatty acids enhances the adsorption of pancreatic lipase to interfaces (*34*). To help understand these effects, our laboratory investigated the interaction of colipase with monomolecular films of diacylphosphatidylcholine, free fatty acid, acylglycerols, and their mixtures (*35*, *36*). Based on data obtained in vivo, but in contrast to most of the earlier studies of PTL−colipase function, colipase−lipid interactions were characterized thermodynamically at a pH consistent with that in the intestinal lumen (*37*) and in the absence of calcium ion (*38*). The results revealed preferential interactions between colipase and non-phosphatidylcholine in both pure and mixed-lipid monolayers. As a consequence of this interaction, it was concluded that the cofactor will laterally concentrate non-phospholipids, such as fatty acids and acylglycerols, in its vicinity. Thus, colipase may serve the additional role in lipolysis of recruiting reactants to the PTL molecule to which it is bound.

As noted above, the presence of phospholipids tends to inhibit PTL adsorption to an interface (*19*). However, in their absence, PTL binds tightly to surfaces comprised of its substrates or products (*39*). Thus, the recently discovered ability of procolipase or colipase to create a reactant-rich interface in its vicinity in the presence of phospholipids suggested that the cofactor might also modulate the affinity of PTL for the interface. To test this hypothesis, the adsorption of PTL to phospholipid−fatty acid monolayers was measured in the presence and absence of colipase. The results, described herein, show that such interactions not only contribute to PTL adsorption but are absolutely required for it to occur.

MATERIALS AND METHODS

13,16-*cis*,*cis*-Docosadienoic acid (DA) was from NuChek Prep (Elysian, MN), [1-^{14}C]oleic acid, 1-palmitoyl-2-[1-^{14}C]-oleoyl-*sn*-glycero-3-phosphocholine, and inorganic [^{32}P]-phosphate were from NEN (Boston, MA), and 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC) was from Avanti Biochemicals (Alabaster, AL). All lipids were shown to be ≥99% pure by thin-layer chromatography. Type 1PS (7 cm diameter) hydrophobic paper for monolayer collection was from Whatman (Clifton, NJ). Before use the papers were cleaned by washing them sequentially with chloroform/methanol (2:1 v/v), chloroform, and hexane, followed by air-drying at 150 °C for 50 min (*40*). Procolipase was purified from porcine pancreas and converted to colipase and to [^{14}C]-colipase with a specific radioactivity of 21 dpm/pmol as reported elsewhere (*35*). PTL (triacylglycerol acylhydrolase, EC 3.1.1.3) was purified from an extract of porcine pancreas using conventional techniques as previously described (*41*). During purification, PTL and colipase activities were measured by using a modification of a pH-stat assay originally described by Momsen and Brockman (*41*). The purified lipase contained less than 1 mol % residual colipase. Protein concentration was determined by the absorbance at 280 nm using an $E^{1\%}$ of 13.3 for lipase (*42*) and 3.6 for colipase (*43*). Using these assays for protein and activity, colipase and lipase were found to have specific activities of 35 500 and 10 300 units/mg, with 1 unit of activity defined as 1 μmol of fatty acid released per minute at 25 °C with tributyroylglycerol as the substrate. For assaying the catalytic activity of colipase dissolved in organic solvent, the usual titrametric assay procedure (*41*) was modified. Aliquots of concentrated colipase in solvent were diluted into the complete, i.e., substrate- and detergent-containing, buffer, rather than detergent-containing buffer, and sonicated for 2 min in a bath sonicator. After 20 min, an appropriate aliquot of this emulsion was added to 3 mL of complete assay mixture in an assay vessel, sonicated for an additional 2 min. The reaction was initiated by the addition of an excess of colipase-free PTL after which the rate of substrate hydrolysis was measured. Control experiments showed that colipase from an aqueous solution was unaffected by the small quantities of solvent dispersed in the substrate emulsion.

All studies of PTL and [^{14}C]colipase adsorption were conducted at 24 °C using an interfacial monitor/controller mounted on a Kinetic Systems (Boston, MA) vibration isolation table. It measures the surface pressure/tension of lipid/protein films by the Wilhelmy method using a Cahn Model D200 microbalance from which was suspended 24 ga Nichrome wire (*44*). Positioning of the microbalance and Wilhelmy wire was controlled by a stepper motor-driven linear transport. Measurements of surface tension and, hence, surface pressure were accurate to within ±0.1 mN/m. The aqueous subphase was contained in a Teflon circular trough (internal diameter = 5.1 cm, volume = 19.5 mL) mounted on a thermoelectric base plate controlled by a Model TC202 Bipolar Temperature Controller (Medical Systems Corp., Greenvale, NY). Stirring of the cylindrical aqueous subphase compartment was achieved with a Teflon-coated magnetic bar (2.5 × 35 mm) driven by a conventional magnetic stirrer at 70 rpm.

To measure PTL adsorption to interfaces, monolayers of SOPC, DA, and colipase were formed by spreading the lipid−colipase mixture onto the aqueous subphase from chloroform/methanol/water (5:5:1) using a 10 μL, gastight digital syringe (Hamilton Co., Reno, NV). The monolayer was equilibrated until the surface pressure was stable, usually about 10 min. To enable later measurement of subphase carryover during monolayer collection, 20 μL of subphase buffer containing ∼4 μCi of [^{32}P]phosphate was introduced into the stirred aqueous subphase from a microsyringe through a small port in the side of the circular compartment. One minute later an aliquot of enzyme solution was added in the same manner and the surface pressure was monitored as a function of time. After 30 min, the monolayer and adsorbed PTL were collected on one side of a circle of hydrophobic filter paper as described (*40*). The paper was placed in a 7.5 cm diameter crystallization dish with the monolayer-containing side facing upward, and 80 mL of assay mixture was immediately added. Assay mixture was an emulsion containing 32.9 mM tributyroylglycerol, 1 mM PIPES, 1 mM $CaCl_2$, 0.02% NaN_3, 0.1% Brij35, 0.15 M

NaCl, and 6.0 mM sodium taurodeoxycholate. In control experiments with 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine, it was shown that 85% of the lipid in the monolayer was recovered on the paper. As recently reviewed, this is a typical monolayer recovery when only one side of the paper is used, as is necessary when protein adsorption is being measured (*40*). All measurements of PTL adsorption to monolayers were corrected for this recovery efficiency. A correction was also made for carryover of PTL in aqueous subphase adherent on the paper. This was accomplished by measuring the amount of [^{32}P]phosphate recovered with the paper and in an aliquot of subphase as described (*40*). Overall reproducibility of measurements of PTL adsorption is estimated at ±15% except for very low levels of adsorption for which the relative error is higher.

RESULTS

To quantitate the effects of colipase on lipase adsorption, colipase must be present in the interface at a known concentration. Prior studies had shown that this could be accomplished by adsorption of the cofactor from the subphase (*36*). However, there appeared to be several disadvantages to that approach for the present study. For each monolayer lipid composition to be used, the isotherm relating the surface concentration of colipase to its bulk concentration would need to be determined. Aside from being time-consuming, such isotherms would be difficult to obtain and interfacial colipase concentration difficult to control. This is because the high affinity of colipase for DA-rich interfaces results in its nearly complete depletion from the subphase at subsaturating concentrations. In pilot experiments, such high affinity, and, hence, low bulk concentrations of colipase, necessitated hours of equilibration to achieve a constant colipase concentration in the interface (W. E. Momsen and H. L. Brockman, unpublished). Additionally, PTL added to a subphase containing colipase would not only adsorb to the interface but also bind to colipase in solution (*17*, *25*, *26*). This would add an additional equilibrium to the system, making it difficult to control the aqueous concentration of free PTL.

A more desirable approach for forming mixed lipid−colipase monolayers was to spread the monolayer components from a volatile organic solvent at the air−buffer interface. This required a solvent in which both the lipids and colipase were soluble at concentrations high enough to spread a 20 cm^2 monolayer from a volume <10 μL and which would result in both the lipids and colipase being quantitatively retained in the monolayer. The possibility that colipase could be solubilized in organic solvents was suggested by a report of its chromatography using an organic solvent mobile phase with retention of its cofactor activity (*45*). After considerable trial and error with mixtures of polar and nonpolar solvents, two were identified which would cosolubilize the lipid and protein constituents at sufficiently high concentration. With one of these solvents, *tert*-butyl alcohol/water (75:25) recovery of [^{14}C]colipase spread to surface pressures from 3 to 24 mN/m was ≤85% of that added. With the other, chloroform/methanol/water (5:5:1), it was found that up to a surface pressure of 17−18 mN/m most of the [^{14}C]colipase spread could be recovered from the interface (filled circles vs dotted line, Figure 1). Addition of more [^{14}C]colipase to the interface resulted in the dissolution of the excess [^{14}C]colipase into the subphase. This result was consistent with prior studies in that a maximum surface pressure of 18−20 mN/m is generated when [^{14}C]-colipase is adsorbed from solution to the argon−buffer interface (*36*). The maximum stable monolayer level of [^{14}C]-colipase of 10 900 cpm shown in Figure 1 corresponds to a [^{14}C]colipase surface concentration of 28 pmol/cm^2. This represents a close-packed monolayer of cofactor at the interface (*35*). In other experiments with a Langmuir film balance (M. M. Momsen, unpublished), it was shown that the surface pressure−area isotherm for colipase spread from this solvent was unaffected by waiting for periods from 4 to 16 min before initiating compression. This indicates the stability of the colipase against dissolution at low surface pressure. It was further determined that colipase, after being stored in the solvent for >45 days, fully retained its ability to serve as a cofactor for PTL.

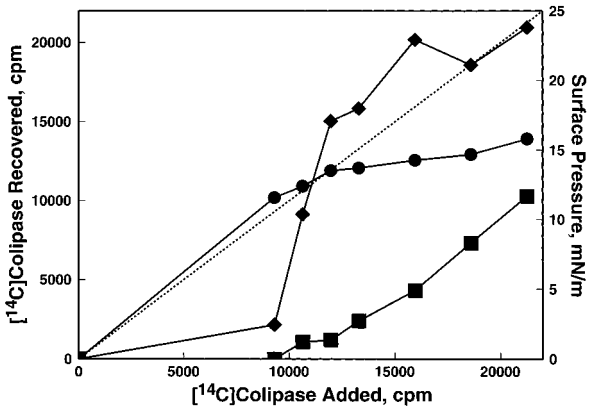

FIGURE 1: Concentration dependence of [^{14}C]colipase retention at the argon−buffer interface. [^{14}C]Colipase recovered in (●) monolayer and (■) subphase 10 min following monolayer formation. (◆) Final surface pressure of the monolayer.

Based on earlier studies (*35*−*37*), it was anticipated that the inclusion of lipids in the monolayer would enhance the retention of colipase with respect to the surface pressure, i.e., lipid packing density, in the monolayer. To test this, mixed monolayers of colipase and either SOPC, DA, or a 65:35 mixture of the lipids were spread to different initial surface pressures. SOPC was used as a model diacylphosphatidylcholine and DA as a reaction product. It should be noted that because no acyl acceptor is present in the system, DA, and hence the interface, remains chemically unmodified in subsequent studies in which lipase was present (below).

Ten minutes after forming the mixed lipid−colipase monolayer, it was collected, as described under Materials and Methods, and its content of [^{14}C]colipase was determined. Figure 2 shows the percentage of spread [^{14}C]-colipase recovered as a function of the initial surface pressure of the monolayer. Up to an initial surface pressure of 20 mN/m (dotted line), essentially 100% of [^{14}C]colipase remained in the monolayer with all lipids tested. At higher surface pressures, however, the type of lipid determined [^{14}C]-colipase retention. As exemplified in Figure 1, [^{14}C]colipase not retained in the monolayer in these experiments was also found in the aqueous subphase (data not shown). With SOPC alone, only 20% of [^{14}C]colipase remained in a monolayer spread to an initial surface pressure of 29 mN/m. In contrast, with DA as the lipid, retention of [^{14}C]-colipase at 30 mN/m was 97%. With the SOPC/DA/

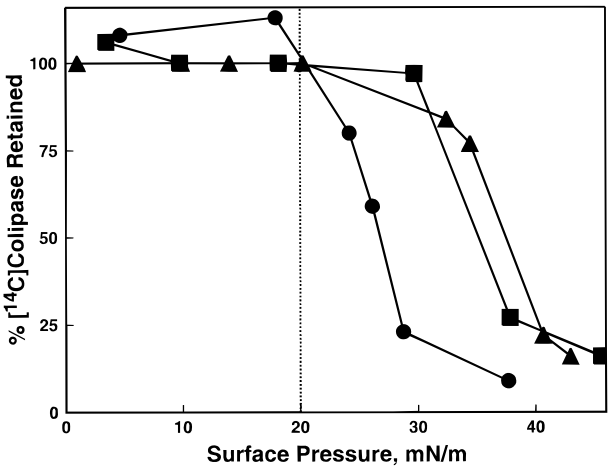

FIGURE 2: Retention of [^{14}C]colipase in lipid monolayers. (●) [^{14}C]-Colipase/SOPC (2.25:97.75), (▲) [^{14}C]colipase/SOPC/DA (1.47:64.05:34.50), (■) [^{14}C]colipase/DA (0.9:99.1).

[^{14}C]colipase (64.05:34.50:1.47) mixture, retention was similar to that observed with DA alone. Other lipid compositions were also tested and gave results consistent with the pattern shown in Figure 2 (data not shown). These surface pressure dependencies of [^{14}C]colipase retention are consistent with results of studies conducted with the same lipids in which [^{14}C]colipase was adsorbed from solution (see Discussion). On the basis of these results, an initial surface pressure of 20 mN/m was selected for determining the role of colipase in regulating PTL adsorption.

For measuring the regulation of PTL adsorption to lipid monolayers containing colipase, it would seem desirable to use a gross excess of PTL in the aqueous subphase to saturate all potential colipase-generated adsorption sites. However, experimentally this approach makes it difficult to measure accurately colipase effects on PTL adsorption. This is because of carryover of PTL from the aqueous phase adherent on the hydrophobic paper used to recover the monolayer for PTL determination. Carryover increases linearly with increasing PTL concentration in the aqueous phase, whereas PTL adsorption saturates at high aqueous PTL (*40*). Under conditions where net PTL adsorption is low but PTL aqueous concentration is high, preliminary experiments (not shown) revealed that most of the PTL activity recovered could be attributed to subphase carryover. Hence, it was necessary to utilize a PTL concentration which would allow measurement of adsorbed PTL but without PTL being excessively carried over from the aqueous phase or depleted from the aqueous phase. The latter condition ensures that aqueous PTL concentration remains essentially constant throughout the range of surface concentrations of PTL to be measured. To determine the appropriate concentration of PTL for this study, an isotherm was determined for PTL adsorption to monolayers of DA initially at 18 mN/m. Based on preliminary observations of the time dependence of surface pressure changes observed following PTL addition to the aqueous subphase under DA monolayers (data not shown), an equilibration time of 10 min was used. Adsorption of PTL to DA, corrected for subphase carryover, was linear to ~30 nM PTL. At 20 nM PTL in our apparatus, adsorption of an entire monolayer of PTL at the interface, should it occur, would result in only a ~20% depletion of bulk phase PTL. On this basis and with the considerations

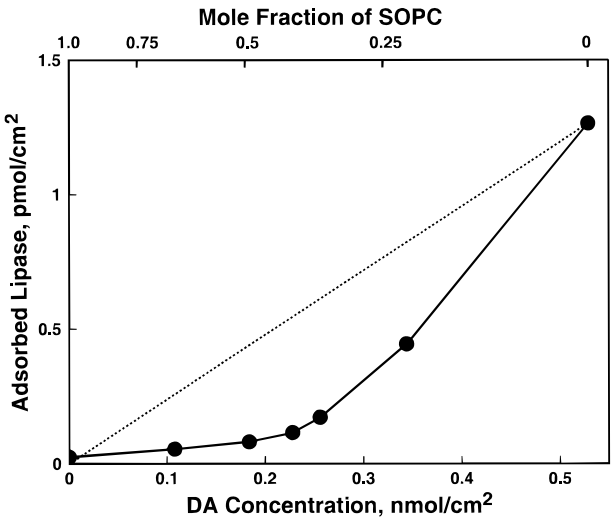

FIGURE 3: Inhibition of pancreatic lipase adsorption to fatty acid monolayers by phosphatidylcholine. The dashed line indicates the expected level of adsorption if all surface occupied by fatty acid were available for lipase adsorption. Incubation time, with stirring, was 10 min, initial surface pressure was 20 mN/m, and PTL was added to the subphase to a concentration of 20 nM.

noted above, a PTL concentration of 20 nM was chosen for measuring the regulation of PTL by colipase and lipids.

In an earlier study documenting the ability of diacylphosphatidylcholine to inhibit PTL-catalyzed reactions in monolayers, the inhibition of PTL adsorption by diacylphosphatidylcholine was not measured directly (*46*). Instead, it was inferred from more detailed studies using pancreatic carboxylester lipase adsorption (*39*) in which both inhibition of catalysis and adsorption were observed. To directly determine the effect of SOPC, PTL adsorption from an aqueous phase containing 20 nM PTL to monolayers of SOPC−DA was determined as a function of monolayer lipid composition at an initial surface pressure of 20 mN/m (Figure 3). It should be noted that SOPC is not a substrate for PTL (*47*). In the figure, the PTL adsorption data are presented as a function of the surface concentration of DA. This is done to illustrate the linear nature of the expected level of PTL adsorption as a function of DA concentration, i.e., the fraction of monolayer area occupied by DA. The ideal line was calculated using the value of 1.26 pmol/cm^2 for PTL adsorption to DA alone shown in the figure (rightmost data point) and the prior observation that molecular areas of SOPC and DA in monolayers, though different, are additive at 20 mN/m (*48*). Figure 3 shows that adsorption of PTL to SOPC alone was negligible. Even at a DA surface concentration of 0.2 nmol/cm^2, which corresponds to a DA mole fraction of ~0.6 in the mixed monolayer, adsorbed PTL remains less than 10% of the maximum value measured with a monolayer of DA alone. Thus, as observed earlier with carboxylester lipase (*39*) and consistent with catalytic measurements of PTL activity in monolayers at comparable aqueous concentration of PTL (*46*), the adsorption of PTL to SOPC-rich monolayers is severely inhibited in SOPC-rich surfaces.

According to the current paradigm (see the Introduction), the presence of colipase in an SOPC-rich interface should increase measured PTL adsorption toward or, possibly, beyond the values indicated by the dotted line in Figure 3. This hypothesis was tested directly by spreading mixtures of SOPC, DA, and colipase to a surface pressure of 18−20

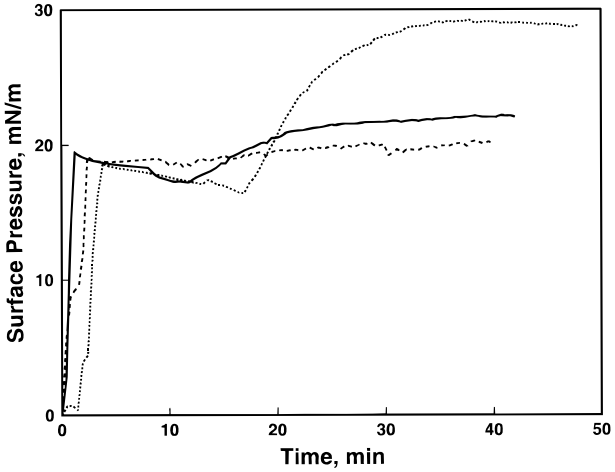

FIGURE 4: Time course of surface pressure changes accompanying pancreatic lipase adsorption to lipid monolayers. Dashed line, SOPC/[^{14}C]colipase (97.74:2.26); solid line, SOPC/DA/[^{14}C]colipase (39.71:59.57:0.73); dotted line, DA alone.

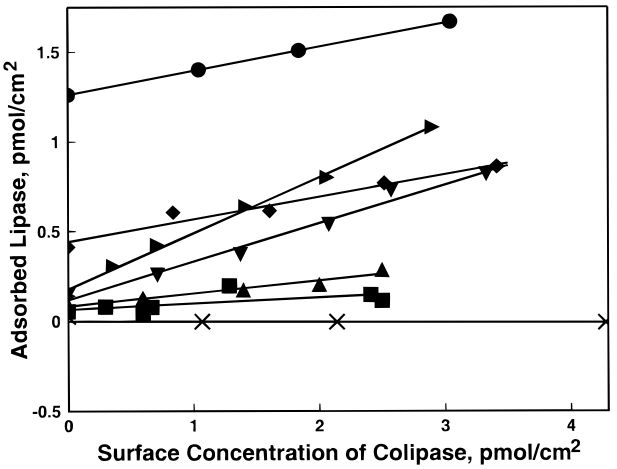

FIGURE 5: Effect of colipase content on pancreatic lipase adsorption to lipid monolayers. Relative SOPC/DA composition: 100:0 (×); 65:35 (■); 50:50 (▲); 40:60 (▼); 35:65 (arrowhead); 20:80 (◆); 0:100 (●). Solid lines are regression lines obtained with each data set.

mN/m and adding PTL to 20 nM in the stirred subphase. The addition of PTL caused changes in the surface pressure of the monolayer as exemplified in Figure 4. There was almost no change with the SOPC monolayer in contrast to a change of 12 mN/m with DA. The traces show that the adsorption was essentially complete after the 30 min incubation period. At that time, the monolayer was recovered and its content of PTL determined. The surface concentration of adsorbed PTL measured with each SOPC/DA/colipase monolayer is given in Figure 5 as a function of the surface concentration of colipase. The maximum changes in surface pressure accompanying the adsorption of PTL to the interface at all compositions and colipase contents are shown in Figure 6. These were as large as 15 mN/m and show an approximately linear correlation with PTL adsorption with a slope of 0.7 mN·m/nmol.

The most obvious and significant result shown in Figure 5 is that in the absence of DA, PTL adsorption does not occur. This absence of adsorption is evident even at the highest colipase surface concentration shown, which corresponds to ∼15% of the monolayer surface area being occupied by colipase. Even when the mole fraction of DA

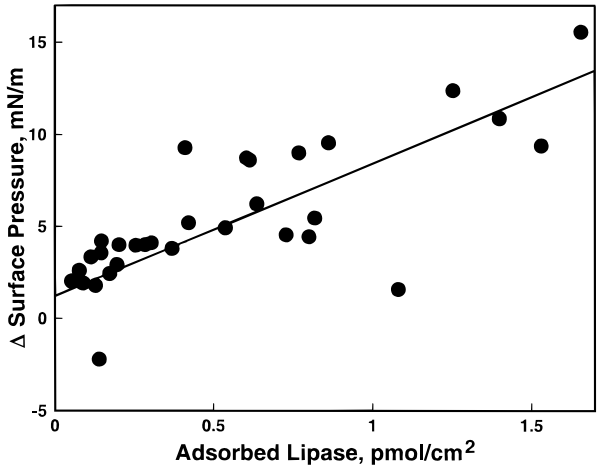

FIGURE 6: Surface pressure changes accompanying pancreatic lipase adsorption to lipid monolayers. Data are from experiments shown in Figure 5. The solid line is a regression line.

in the lipid monolayer was increased to 0.35, the increase in PTL adsorption above that occurring in the absence of colipase was small. At higher mole fractions of DA, a greater enhancement by colipase is observed, but PTL adsorption in the absence of colipase also becomes significant. The latter quantity is indicated by the value of each data set on the ordinate. Within error, at each lipid composition used, the enhancement of PTL adsorption by colipase is linear with colipase surface concentration, as indicated by the regression lines in Figure 5. Thus, in this lower, more physiological range of colipase concentrations, the ability of the cofactor to enhance or enable PTL adsorption depends on monolayer composition in an apparently complex manner. Additional measurements of PTL adsorption were made at higher colipase concentrations (not shown). At a monolayer content of colipase around 5 mol %, which approaches 50% of monolayer area, enhancement of PTL adsorption was maximal. However, measurements of PTL adsorption in this range were highly variable for reasons not presently understood.

One possible explanation for the relative lack of any colipase enhancement of PTL adsorption at low DA mole fractions (Figure 5) is that PTL may catalyze the dissolution of colipase from the interface under the conditions used. This hypothesis was tested by performing experiments identical to those described above but replacing colipase with [^{14}C]-colipase (∼3.5 mol %) in monolayers of SOPC and DA and omitting [^{32}P]phosphate from the aqueous phase. At the end of the 30 min exposure to 20 nM PTL in the subphase, the monolayer was collected and its content of [^{14}C]colipase determined by scintillation counting. A 1.0 mL aliquot of the subphase was also collected for determination of [^{14}C]-colipase concentration. As shown in Table 1, in the presence of PTL recovery of [^{14}C]colipase in the monolayer was 80−90% with both pure SOPC and 0.65 DA relative to SOPC + DA under all conditions. Considering the small absolute number of radioactive counts used in these experiments and the errors inherent in measurements of this type, the recoveries under all conditions are identical. Thus, the relative lack of enhancement of PTL adsorption by interfacial colipase shown in Figure 5 for SOPC-rich monolayers cannot be attributed to PTL-induced desorption of [^{14}C]colipase from the spread lipid−[^{14}C]colipase monolayer.

Table 1: Retention of [^{14}C]Colipase in SOPC/DA Monolayers

SOPC:DA:[^{14}C]-colipase ratio	[lipase] (nM)	cpm added	cpm recovered, %	
			monolayer	subphase
0.965:0:0.035	−	2326	88	4.0
0.965:0:0.035	20	2115	91	4
0.628:0.338:0.035	−	2138	76	−
0.628:0.338:0.035	20	2206	91	−
0:0.965:0.035	−	2460	83	−
0:0.965:0.035	20	2316	80	11

Kinetic factors might also prevent colipase-facilitated adsorption of PTL to SOPC−colipase monolayers. It can be speculated that the known 1:1 PTL−colipase complex must form in solution (*17*, *25*, *26*) before PTL and its accompanying colipase can be adsorbed to SOPC monolayers on a reasonable time scale. This could help stabilize the flap of PTL in the open position and enhance the rate at which the complex can adsorb to the interface. To test this possibility, PTL and colipase were mixed in a 1:1 ratio at a PTL concentration of 7.3 μM and incubated for >24 h at 4 °C to facilitate formation of the PTL−colipase complex. This mixture was then injected beneath a monolayer of SOPC at 20 mN/m following the protocol described above to yield a final PTL concentration of 20 nM. After 30 min, the monolayer was collected and the surface concentration of PTL determined. It was only 0.04 ± 0.03 (n = 6) pmol/cm^2, a value close to the values shown for PTL adsorption to preformed SOPC−colipase monolayers (Figure 5). Thus, premixing of PTL and colipase in the aqueous phase did not kinetically enhance colipase-mediated PTL adsorption to SOPC monolayers. During these experiments it was noted that the surface pressure increased from the initial value of 20 mN/m to ∼27 mN/m, suggesting adsorption of colipase to the monolayer was occurring. To determine if this was the case, colipase was replaced with [^{14}C]colipase, and the experiment was repeated with PTL and [^{14}C]colipase pre-incubated at 7.2 μM and, again, injected to a concentration of 20 nM each into the aqueous phase. The surface concentration of [^{14}C]colipase subsequently recovered in the monolayer was 1.17 ± 0.25 (n = 7) pmol/cm^2, a value (coincidentally) comparable to that spread with SOPC and DA to obtain the data shown in Figure 5. Thus, significant adsorption of colipase from the PTL−[^{14}C]colipase mixture in the subphase to the SOPC monolayer occurs even in the absence of significant PTL adsorption.

DISCUSSION

In the absence of colipase, the adsorption of PTL to DA shows a value of 1.26 pmol/cm^2 at 20 nM PTL (Figure 3), and at 112 nM PTL, apparent saturation is observed at ∼5 pmol/cm^2 (*46*). This latter value is equivalent to about 60% of that expected if the protein were globular and hexagonally close-packed. That so much native lipase protein can adsorb to a lipid monolayer precludes the enzyme being adsorbed between the lipid molecules, i.e., "penetrating" the monolayer. Rather, PTL must reside essentially adjacent to the lipid monolayer. The progressive replacement of DA with SOPC (right to left in Figure 3) leads to greatly diminished adsorption of PTL compared to that expected on the basis of composition alone (dotted line, Figure 3). We have suggested that such inhibition is a consequence of the surface not having, at any instant in time, a sufficient fraction of surface area occupied by appropriate adsorption sites for PTL (*46*). Apparently, for productive lipase adsorption in the absence of colipase, DA sites must be sufficiently devoid of SOPC and large enough to accommodate the enzyme.

In contrast to PTL, we have observed recently that either form of its cofactor, procolipase (*37*) or colipase (*36*), competes with lipid acyl chains for monolayer occupancy. Both forms of the cofactor interact preferentially with substrates, e.g., diacylglycerol, and products, e.g., DA, of lipolysis, as compared with SOPC (*36*, *37*). A consequence of such preferred interaction is that lipase substrates and products are concentrated in the vicinity of colipase. Because PTL adsorption appears to require a "nanodomain" of relatively SOPC-free surface, the properties of procolipase and colipase suggested that they might enhance the formation of PTL adsorption sites in mixed DA−SOPC interfaces and, thereby, enhance PTL adsorption. On the other hand, crystallographic data (*11*) and solution studies (*17*, *25*, *26*) clearly show direct, protein−protein interactions between PTL and colipase to form a 1:1 complex. Thus, the presence of colipase in an interface could be sufficient to enable or enhance PTL adsorption.

Essential to differentiating these possible roles for colipase in regulating PTL adsorption was having the ability to prepare monolayers of known lipid and colipase composition. This was accomplished, as described herein, by identifying an organic solvent in which both lipids and colipase were mutually soluble at concentrations sufficient for forming monolayers without excessive solvent addition. Importantly, colipase so solubilized retained its activity when returned to an aqueous milieu. Overall, the results obtained with spread monolayers containing [^{14}C]colipase in the presence and absence of lipids are similar to those obtained earlier under conditions of apparent thermodynamic equilibrium (*35*−*37*). This shows that colipase-containing monolayers spread from organic solvent are a valid model system with which to study the regulation of PTL adsorption by colipase.

If the role of colipase were solely to provide a binding site for lipase via protein−protein interactions, its mere presence in an interface should be sufficient to enable or enhance PTL adsorption. Inspection of Figure 5 shows that this is not the case. No measurable adsorption of PTL occurs in the absence of DA, and even with 0.35 DA in the interface, relative to SOPC+DA, colipase has little effect. Higher mole fractions of DA did, however, result in significant enhancement of PTL adsorption in an apparently complex manner. Control experiments showed that the inability of colipase to enable PTL adsorption did not result either from a loss of colipase from the interface (Table 1) or from a need for a preformed PTL−colipase complex in the aqueous phase. Thus, the data in Figure 5 and the controls show that in an SOPC-rich interface the presence of DA is required for colipase to enhance PTL adsorption. These observations support the notion that colipase may be creating "nanodomains" of DA about itself which facilitate PTL adsorption.

Quantitating the extent to which colipase regulates the "quality" of the interface with respect to facilitating PTL adsorption is difficult. This is because the term "quality", as frequently used in describing the regulation of lipolysis, has no quantitative meaning or proper frame of reference. However, because the experimental system in the present

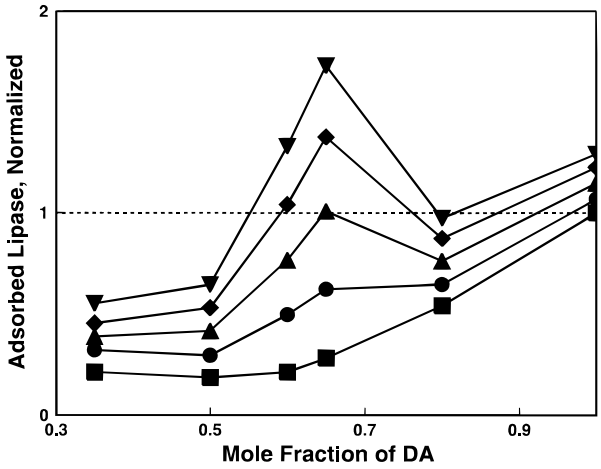

FIGURE 7: Normalized adsorption of pancreatic lipase to colipase-containing lipid monolayers. Data are taken from Figure 5 at arbitrary colipase concentrations of 0 (■), 0.7 (●), 1.4 (▲), 2.1 (◆), and 2.8 (▼) pmol/cm^2 and normalized for DA content using the dotted line in Figure 3 as described in the text. The dotted line at 1.0 indicates expected adsorption to DA alone in the absence of colipase.

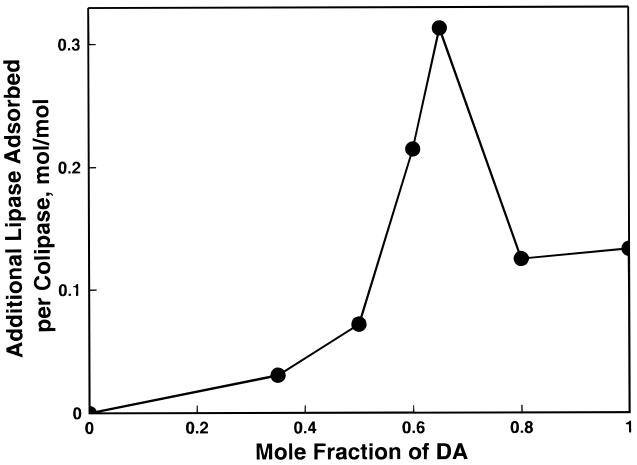

FIGURE 8: Lipid composition dependence of the enhancement of pancreatic lipase adsorption to lipid monolayers by colipase. Data points are the slopes of the regression lines shown in Figure 5.

study is so well-defined and the data sets in Figure 5 are reasonably linear, some quantitation is possible. One approach is to compare the overall "quality" of the interface at differing lipid compositions and colipase contents. To do this, it is necessary first to normalize the data in Figure 5 with respect to the surface concentration of DA in the interface. This corrects for the fact that at, for example, 0.35 DA there is a much smaller fraction of the interfacial area occupied by DA than at a DA mole fraction of 1.0. Values of adsorbed PTL at several, arbitrary colipase concentrations (Figure 5) were tabulated as a function of DA mole fraction relative to DA + SOPC. This is equivalent to drawing vertical lines in Figure 5 at selected, but arbitrary, colipase contents to obtain the data values at which each line intersects each of the regression lines drawn through the data. Each data point was then divided by the relative ideal PTL adsorption value at that mole fraction. For each mole fraction of DA used, this normalization factor was taken as the corresponding value from the dotted line in Figure 3 divided by the value of 1.26 pmol/cm^2 obtained with pure DA, i.e., the fraction of surface area covered by DA. The normalized adsorption data at the selected concentrations of colipase are shown in Figure 7 as a function of the DA mole fraction relative to SOPC + DA. The horizontal dotted line in the figure indicates a relative level of PTL adsorption of 1.0, i.e., that observed with DA in the absence of colipase. The figure shows that in the presence of colipase the ability of DA to support PTL adsorption shows a maximum around 0.6 mole fraction of DA and is a direct function of the colipase concentration. For the higher colipase concentrations, the maximum normalized adsorption values approach 2.0. This means that, with sufficient colipase, there is almost twice as much PTL adsorbed per unit area of the surface which is occupied by DA, than to DA alone in the absence of colipase or SOPC. This apparent increase in the "quality" of the DA surface is also consistent with crystallographic data (*49*) which show that protein−protein interactions between PTL and colipase help to stabilize the flap in the opened position, exposing hydrophobic residues. These presumably participate in the adsorption of PTL to DA concentrated about colipase.

Although the above analysis compares the "quality" of DA to support PTL adsorption at different lipid compositions, it does not separate PTL adsorption mediated by colipase from that which occurs in its absence. This is better accomplished by examination of the slopes of the lines shown in Figure 5. These slopes, which have dimensions of moles of PTL adsorbed per mole of colipase, are plotted in Figure 8 as a function of the DA mole fraction relative to SOPC. Between 0 and 0.65 DA, the enhancement increases from essentially 0 to a value of 0.31 in an apparently exponential manner. However, at DA $\geq$0.8, the enhancement is approximately half of the maximum. Comparison of Figure 8 with Figure 3 suggests that the drop in colipase enhancement of PTL adsorption at higher DA mole fractions is largely a result of the ability of DA-rich interfaces to support PTL adsorption in the absence of colipase. At high mole fractions of DA, this "unassisted" adsorption effectively competes with colipase-assisted adsorption and, thereby, lowers the relative enhancement. At lower mole fractions of DA, the exponential dependence of PTL adsorption (Figure 8) shows clearly the importance of having DA present in the interface for colipase to enable PTL adsorption. This dependence is consistent with the ability of colipase to concentrate nonphospholipids, like DA, in its vicinity. As the DA:colipase ratio increases, the data suggest that a larger proportion of colipase molecules have sufficient DA around them to support PTL adsorption. Thermodynamic measurements indicate that each colipase molecule can effectively concentrate 20−30 molecules of DA in its vicinity (*36*). If, to a first approximation, the interaction of PTL with DA dominates the binding energy, then the work of adsorption should then be the same for each PTL molecule, regardless of monolayer lipid composition or the presence of colipase. This conclusion is supported by Figure 6 which shows, to a first approximation, that the surface pressure increases vary linearly with PTL adsorption for all experiments represented in Figure 5. Thus, analysis of the enhancement of PTL adsorption by colipase as a function of interfacial lipid composition supports the idea that the cofactor assists PTL adsorption by preforming DA-enriched adsorption sites.

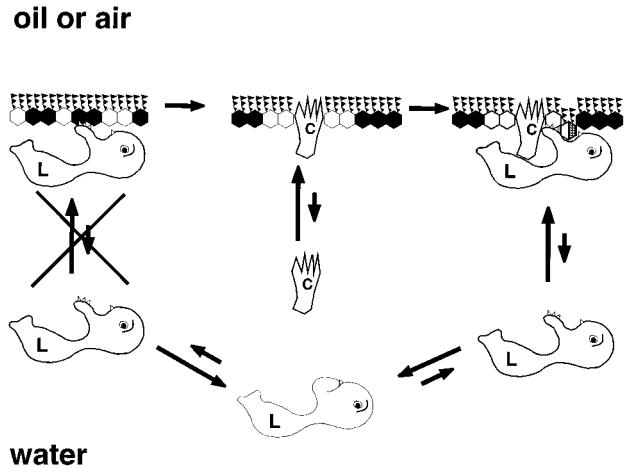

FIGURE 9: Model for colipase (C) enhancement of the adsorption of pancreatic lipase (L) to phosphatidylcholine (filled hexagons)−reactant (open hexagons) interfaces.

This study utilized only DA as the non-phospholipid for studying the regulation of PTL adsorption to SOPC-containing monolayers. DA was chosen primarily to prevent PTL from catalyzing changes in the lipid species composition and abundance in the interface during the measurement of adsorption. Although DA is a product of lipolysis, it is also a substrate. It participates in the back-reaction of ester synthesis and can undergo PTL-catalyzed ^{18}O exchange between DA and water in the absence of a lipid acyl acceptor (*46*). Moreover, the rate constant for the PTL-catalyzed oxygen exchange reaction is similar to that for diacylglycerol hydrolysis (*46*). In its interactions with colipase, DA also shows essentially the same behavior as ester substrates of PTL (*36*). These similarities suggest that the results obtained in this study will apply to all substrates and products, collectively reactants, of lipolysis.

The hypothesis suggested by the data obtained in this study is shown pictorially in Figure 9. Reactant molecules, in the more general sense which includes fatty acids, alcohols, and ester substrates, are indicated by open hexagons and phosphatidylcholine by filled hexagons. The left-hand section of the monolayer depicts the inability of PTL to adsorb to a reactant-containing but phosphatidylcholine-rich interface. The center monolayer section depicts the rearrangement of the lipid molecules from the left-hand monolayer induced by the adsorption of colipase from the aqueous phase. This colipase-reactant nanodomain then serves as the PTL adsorption site and allows catalysis to occur (right-hand panel). Not shown in the figure for clarity is PTL−colipase binding in the aqueous phase to preform a 1:1 complex nor the possible separate interaction of PTL and colipase with micelles (*50*). This complex can, presumably, bind directly to the surface with concomitant lateral rearrangement of the lipids. The present study did not address adsorption of the preformed PTL−colipase complex except in one set of control experiments. It is not known which path predominates in vivo.

The model shown in Figure 9 can qualitatively explain past observations concerning the interaction of PTL with interfaces and its regulation by reactants. For example, there is a kinetic lag in the PTL-catalyzed hydrolysis of phosphatidylcholine-stabilized triacylglycerol emulsions and mixed monolayers (*8*, *34*). This lag is dramatically shortened in the presence of colipase or upon hydrolysis of the phospholipid. The lag arises because initially, in the absence of colipase or hydrolysis products, few lipase molecules can bind to the interface (*8*, *34*). This is because only a few mole percent of the triacylglycerol is soluble in the largely phospholipid interface (*51*). The PTL adsorbed to these few sites will slowly generate reaction products such as diacylglycerol and DA. In the absence of colipase, lag times are very long because products must accumulate globally to exceed the critical concentration needed to support continued hydrolysis [(*46*) and references cited therein]. Once this threshold is crossed, hydrolysis becomes autocatalytic. In the presence of colipase, amphipathic reactants facilitate interaction of the cofactor with the interface and become concentrated about the colipase molecules (*36*). As the colipase−reactant nanodomains are formed, they create adsorption sites for PTL (e.g., Figure 8) that would not be present at the same global reactant concentration in the absence of colipase. The PTL adsorbed to these sites catalyzes the hydrolysis of more substrate, and, hence, the autocatalytic phase of hydrolysis begins at a lower global extent of hydrolysis. In summary, it was recognized many years ago that the reactants of lipolysis were involved in the colipase-mediated adsorption of PTL to phospholipid-rich interfaces (*8*, *12*, *22*, *33*, *34*). The results of this study suggest a mechanism for how the preferential interactions of colipase with the reactants can enhance PTL adsorption and, ultimately, substrate hydrolysis.

In vivo, the gastric predigestion of lipids is believed to render substrate-containing emulsion particles susceptible to PTL-catalyzed lipolysis in the intestine by generating fatty acids and diacylglycerol (*52*, *53*). These are much more surface active than triacylglycerols and, hence, will be present in higher abundance than triacylglycerols on the emulsion surface. This will enable PTL−colipase to become rapidly activated, as described above, when mixed with gastric contents in the duodenum. In the intestine, the presence of millimolar concentrations of bile salts ensures the rapid removal of the ultimate products of lipolysis, monoacylglycerol and fatty acid. Thus, as an emulsion particle becomes depleted of tri- and, in particular, diacylglycerols, its surface may become enriched in phospholipid, an abundant constituent of bile. The experiments presented in this work showed that a phospholipid surface devoid of reactants cannot support PTL adsorption, even at the relatively low surface pressure of 20 mN/m used. Colipase adsorption affinity for phospholipid is also weaker in the absence of reactants (*36*). If phospholipid hydrolysis is relatively slow compared to glyceride hydrolysis and product removal, a relative abundance of intact diacylphospholipid could lead to desorption of the PTL−colipase complex or its individual constituents. This would provide a mechanism by which lipase avoids becoming trapped in reactant-depleted interfaces.

ACKNOWLEDGMENT

We thank W. E. Momsen for the purification and assay of the proteins used in this study and M. M. Momsen for editorial assistance.

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BI973015R