USING SHORT-CHAIN LECITHINS TO STUDY THE SURFACE BEHAVIOR OF LIPOLYTIC ENZYMES

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Detailed mechanistic studies of lipases and phospholipases are hampered by poorly defined surfaces. In particular, manipulation of lipid component concentrations can drastically affect particle structure and/or enzymatic activity. Short-chain lecithin molecules are a particularly useful building block of complex but characterizable micellar particles. These lecithins, with four to eight carbons in the fatty acyl chains, exist as monomers in aqueous solution and form micelles above a critical micelle concentration (c.m.c.). Their detergent properties allow them to solubilize large fractions of lipophilic substances such as triglycerides (0.5 mol fraction) and cholesterol (0.18 mol fraction). We have used ^{13}C NMR spectroscopy of these lecithins to (*a*) examine the conformation and motion of individual *sn*-1/*sn*-2 chains for monomeric and micellar lecithin; (*b*) assess the conformational effects of chemical changes in the lecithin molecule; and (*c*) characterize mixed particles consisting of short-chain lecithins, triglyceride and/or cholesterol that mimic lipoprotein structure. The characterized particles are then used in kinetic studies of lipolytic enzymes.

RESULTS

In contrast to results with long-chain lecithins, all carbons are resolved in the ^{13}C NMR spectra (1). The extent of *sn*-1/*sn*-2 chain carbon magnetic nonequivalence reflects the aggregation state of the short-chain lecithin. Motional gradients of the fatty acyl chains in micellar lipid show characteristics of lecithins in bilayers: a plateau of "restricted" motion near the glyceryl backbone, then an increased motion in the last two carbons. However, ^{13}C NMR and Raman temperature dependence studies show that the individual molecules in the micelle are quite fluid (2). The monomer-to-micelle ^{13}C chemical shifts can be understood as the result of a solvent change to a hydrogen-bond donor-deficient environment with only a slight increase in *trans* conformers (5–7%) and possibly a small conformational change in the glyceryl backbone. This picture is useful in eliminating several explanations for the interfacial activation characteristics of soluble phospholipases.

The substitution of an ether for an ester linkage yields a lecithin analogue with a slightly lower c.m.c. but similar conformation (3) as judged by NMR parameters. Therefore, ether and ester lipids form an ideal mixed micelle system for kinetic analyses of phospholipases. Our kinetics (Table I) show that ether lecithins are good inhibitors of phospholipase A_2 (*Naja naja naja*), but are neither substrate nor inhibitor for phospholipase *C* (*Bacillus cereus*). As well as indicating that the carbonyl ester is an important binding determinant for phospholipase *C*, this result makes mixed micelles of short-chain ester and ether lecithins useful for studies of surface dilution effects without complications of dilutor-enzyme interactions.

Short-chain lecithin mixed micelles with triglyceride (4) are substrates for pancreatic lipase. By proper choice of triglyceride and lecithin chain lengths all chain carbon atoms in mixed particles can be observed in the ^{13}C NMR spectra; hence motions of each component can be investigated. NMR and phospholipase kinetics suggest that the triglyceride is mostly in a core surrounded by phospholipid. Lipase hydrolysis rates of the triglyceride in these small, relatively homogeneous particles are 0.3–0.5 times those of triglyceride emulsions alone. Once sufficient lecithin is added to solubilize the triglyceride, the reaction rate becomes independent of the lecithin:triglyceride ratio. This particle-specific activity depends on both triglyceride

TABLE I
PHOSPHOLIPASE ACTIVITY TOWARD 5mM DIHEPTANOYL-PC (DiC_7PC) AND MIXED MICELLES WITH DIHEPTYL-PC (e-PC)

Sample	Phospholipase-A_2	Phospholipase-C
	($\mu mole\ min^{-1}\ mg^{-1}$)	
DiC_7PC (5mM)	213(3)	1650(90)
+e-PC (2mM)	81(2)	2000(240)
+e-PC (5mM)	46(3)	1560(10)
e-PC (5mM)	0	<3

TABLE II
PANCREATIC LIPASE ACTIVITY AGAINST OPTICALLY CLEAR SHORT-CHAIN LECITHIN/TRIGLYCERIDE PARTICLES

Sample*	Particle specific activity
	($\mu mole\ min^{-1}\ mg^{-1}$)
DiC_6PC/TB	53(1)
DiC_7PC/TB	70(1)
DiC_8PC/TB	97(9)
TB emulsion	210(40)
DiC_7PC/TH	18(1)
DiC_8PC/TH	10(0.1)
TH emulsion	34(13)

*Assays contain 5mM TB (tributyrin) or TH (trihexanoin), 5mM $CaCl_2$.

and lecithin chain length (Table II). A detailed model is proposed to account for this behavior.

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ASSEMBLY OF THE PROTHROMBINASE COMPLEX

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The term "prothrombinase" refers to the complex composed of the enzyme Factor X*a*, the cofactor protein Factor V*a*, calcium ions and phospholipid, which is thought to be responsible for the physiological conversion of the zymogen prothrombin to the enzyme thrombin. Although Factor X*a* is sufficient for prothrombin activation, the cofactors increase the rate of thrombin generation ~ 300,000-fold at plasma concentrations of enzyme and substrate. Both the enzyme (Factor X*a*) and the substrate (prothrombin) of this reaction are vitamin K-dependent proteins, each of which possesses γ-carboxyglutamic acids at the NH_2 terminals of their respective peptide chains. As a consequence of these residues, these proteins can, in the presence of calcium, bind membranes containing acidic phospholipids. The cofactor for the reaction, Factor V*a*, is present in plasma in the form of a "pro" cofactor species, Factor V. Limited proteolysis by thrombin converts the "pro" cofactor, Factor V, to the active cofactor, Factor V*a*. Current data suggest that Factor V possesses some minimal level of activity, but that Factor V*a* is at least 400 times more active than the uncleaved Factor V.

The "pro" cofactor species, Factor V, is a rather unusual plasma protein. It is a single-chain protein with a molecular weight of 330,000 (1). Both sedimentation and gel filtration analysis of Factor V suggest that the protein is highly asymmetric. The sedimentation coefficient for Factor V is ~ 9.2S and the Stokes Radius is 91–93 Å (1, 2). Factor V possesses three binding sites for calcium, two having dissociation constants of 6×10^{-5}M, one of $< 10^{-8}$M (3). This very tightly-bound calcium appears to have structural significance with respect to the ultimate association of Factor V*a* polypeptide chains. Thrombin cleaves Factor V at at least four points in the polypeptide chain, leading to a Factor V*a* molecule composed of two to three peptide units. Two peptides with molecular weights of 94,000 and 74,000 are essential for the cofactor activity present in Factor V*a* preparations (3, 4). A third peptide, with a molecular weight 31,000, is also associated with the Factor V*a* molecule, while two activation peptides with apparent molecular weights 70,000 and 90,000 are not associated with Factor V*a*. The treatment of Factor V*a* with EDTA permits dissociation of the two essential Factor V*a* chains (94,000 and 74,000), neither of which possesses activity in its own right (5, 6). These chains can be reassociated by the readdition of calcium ion. The functions of the remaining polypeptide chains in Factor V*a* have not yet been investigated. The Stokes Radius obtained on gel filtration of Factor V*a* is ~ 51 Å (2).

Both Factor V and Factor V*a* bind to phosphatidylcholine-phosphatidylserine vesicles and to blood platelets (7, 8). In contrast to the vitamin K-dependent proteins, Factor V binding to membranes is metal ion and ionic strength independent. The activated cofactor binds to acidic phospholipid-containing vesicles with approximately ten times the affinity of either prothrombin or Factor X*a*, and binds to a limited number of sites on the platelet membranes with a dissociation constant of 10^{-10} M.

RESULTS AND DISCUSSION

We are examining the participation of each of the three proteins, phospholipid and platelet membranes, and calcium ions in the generation of thrombin from prothrombin. Our approaches include equilibrium binding measurements of each of the species in the reaction system, using light scattering, fluorescence, and sedimentation techniques, and studies of the conversion of prothrombin to thrombin using both a synthetic substrate and prothrombin (9). In the latter case, the fluorescent inhibitor, dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) has been used as a probe. This compound is a specific inhibitor of thrombin ($K_d = 4 \times 10^{-8}$M) and binds reversibly at the active site in 1:1 stoichiometry (10). The bound probe exhibits fluorescence properties allowing for continuous assessment of the formation of thrombin from a zymogen prothrombin.

In addition to equilibrium studies of biomolecular association processes, we have recently extended our stud-

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