Acrosin is an extrinsic membrane proteinase from spermatozoa which functions in the fertilization process. Liposomes were utilized as a model system to determine possible effects of membrane association on acrosin's enzymatic activity. By comparison with solubilized enzyme, liposome-bound acrosin had a substantial reduction in the apparent affinity for "progressive" inhibitors such as leupeptin, lima bean trypsin inhibitor, soy bean trypsin inhibitor, and for a proteinase inhibitor from sperm extracts. In contrast, the liposome-bound and -solubilized enzymes were essentially identical with respect to the binding of benzamidine and p-aminobenzamidine which are competitive acrosin inhibitors. These results suggest membrane association can influence some but not all of acrosin's enzymatic properties.

Acrosin (EC 3.4.21.10) is a "trypsin-like" serine proteinase (1) extrinsically associated with membranes of the mammalian sperm acrosome (2–11), an intracellular organelle. The ability of exogenous acrosin inhibitors to prevent both in vitro and in vivo fertilization (reviewed by Ref. 12) has stimulated interest in the development of agents for specific acrosin inhibition as well as interest in mechanisms of acrosin regulation. While a large number of acrosin inhibitors have been identified (reviewed by Refs. 13, 14), little is known concerning the effectiveness of the inhibitors against acrosin that is in the membrane-associated state. Brown and Hartree (10) have provided preliminary data which suggest that sperm-associated and -solubilized acrosin differ with respect to inhibition by several endogenous and exogenous inhibitors. Unfortunately, a more detailed study of the sperm-associated enzyme has not been feasible since the presence of multiple forms of active acrosin (14), the inactive zymogen proacrosin (14, 15), endogenous acrosin inhibitors (16), other hydrolytic enzymes (12), as well as many unknown membrane and cytoplasmic components severely limit meaningful interpretations of such experimental data.

An alternate approach to the study of acrosin-membrane interactions has been the utilization of liposomes as a model membrane system with highly purified enzyme. Acrosin was shown to reversibly associate with anionic phospholipid bilayer surfaces through apparent electrostatic charge interactions without any significant effects on enzymatic hydrolysis of either ester or soluble protein substrates (17). In contrast, this report presents data indicating that substantial differences exist between liposome-associated and -solubilized acrosin with regard to inhibition by both an endogenous and several exogenous progressive proteinase inhibitors.

**EXPERIMENTAL PROCEDURES**

Highly purified mg-acrosin was prepared from acid extracts of washed porcine spermatozoa by established procedures (18). Benzamidine, p-aminobenzamidine, BzArgOEt, leupeptin were obtained from Sigma; egg PC and PG were from Avanti Polar Lipids (Birmingham, AL); and LBI, SBI, and bovine trypsin were purchased from Worthington. LBI was further purified by gel filtration on a Sephadex G-50 column (0.9 × 55 cm) in 1 M HCl. A partially purified acrosin inhibitor from sperm extracts was prepared by sequential gel filtration and ultrafiltration as previously described (19). Protein concentrations were estimated spectrophotometrically at 280 nm (1).

Multilamellar liposomes were prepared by dispersing dried mixtures of PC and PG at a 65:35 molar ratio in buffer, through Vortex agitation as previously described (17). Phospholipids were quantitated by phosphate analysis (20) and monitored for purity by thin layer chromatography (17).

Acrosin esterolytic activity was assayed spectrophotometrically by following the hydrolysis of BzArgOEt at 253 nm (14, 21). The assays were performed at 24 °C in either 1- or 3-mL reaction volumes consisting of 0.5 mM BzArgOEt, 0.02 mM Hapes, and 0.1 mM NaCl at pH 8.0. A molar absorption difference of 1.150 M⁻¹ cm⁻¹ was used to convert the change in optical density to micromoles of BzArgOEt hydrolyzed (22).

**Inhibitor Assays—**Dissociation constants (Kᵢ) for benzamidine and p-aminobenzamidine were determined according to the method of Dixon (23) as previously described (24). Acrosin (0.5 μg) was preincubated with or without 0.1 μmol of phospholipid (PG:PG, 65:35) in 0.21 ml of 0.02 M Hapes, 0.1 M NaCl, pH 8.0 at 24 °C. After 5 min, a 0.2-ml aliquot was withdrawn and assayed in 2.5 ml of buffer containing BzArgOEt and inhibitor. At least six different inhibitor concentrations were used over a range of 1 × 10⁻⁸ to 1 × 10⁻³ M for benzamidine and 5 × 10⁻⁷ to 5 × 10⁻⁴ M for p-aminobenzamidine.

Progressive inhibitors were evaluated by following the loss of acrosin activity over time and by determining ISO values, where ISO represents the inhibitor concentration required to reduce the enzyme activity to 50% of the initial uninhibited activity at a constant enzyme concentration and a constant incubation period. For time course evaluations, acrosin (3.2 μg) was preincubated with or without 0.8 μmol of phospholipid (PC:PG, 65:35) in 7.92 ml of 0.02 M Hapes, 0.1 mM NaCl, pH 8.0 at 24 °C. After 5 min, a 0.08-ml aliquot of either 2 μM LBI, 3 μM SBI, or 5 μM leupeptin was added and 1-ml aliquots of the incubation mixture were assayed at intervals by adding 0.01 ml of 0.05 M BzArgOEt.

For ISO determinations, acrosin (0.3 μg) was preincubated with or without 0.1 μmol of phospholipid in 0.9 ml of 0.02 M Hapes, 0.1 mM NaCl, pH 8.0 at 24 °C. After 5 min, a 0.1-ml aliquot of buffer containing appropriate concentrations of inhibitor was added, the mixtures were incubated for 60 min, and then assayed by adding 0.01 ml of 0.05 M BzArgOEt.

1 The abbreviations used are: BzArgOEt, Nα-benzoyl-L-arginine ethyl ester; leupeptin, N-acetyl-L-leucyl-L-leucyl-L-argininal; LBI, lima bean trypsin inhibitor; SBI, soy bean trypsin inhibitor; PC, phosphatidylcholine; PG, phosphatidylglycerol; Hapes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Acrosin inhibition of liposome-bound and solubilized acrosin.

When highly purified acrosin was incubated with leupeptin, LBI, or SBI, a progressive reduction in enzymatic activity was observed, with an initial rapid phase and a prolonged slow phase of increasing inhibition (Fig. 1). Equivalent quantities of liposome-bound acrosin were also inhibited in a progressive manner but at a slower rate and to a lesser extent, as shown in Fig. 1. For example, after a 20-min incubation with leupeptin, LBI, and SBI, solubilized acrosin was inhibited by 71, 87, and 96% while the liposome-bound enzyme was inhibited by 29, 25, and 52%, respectively.

Similar results were observed with an endogenous acrosin inhibitor from sperm extracts. As shown in Fig. 2, after a 10-min incubation, the solubilized enzyme was inhibited by over 99% while the liposome-bound enzyme was inhibited by only 49%. Unfortunately, sufficient quantities of this inhibitor were not available for a more detailed analysis.

Binding and dissociation constants for progressive inhibitors are extremely difficult to determine since a steady state of equilibrium is achieved at slow rates and the binding is often extremely "tight," behaving in an apparently irreversible manner (25, 27). Accordingly, \( K_d \) values were determined to provide a quantitative parameter for comparison of the liposome-bound and solubilized enzyme. In each case, the enzyme was incubated with inhibitor for at least 60 min, a point where further loss of enzymatic activity through inhibition was minimized. Longer incubation periods were not considered feasible since there was a loss in acrosin activity due to autoproteolysis (17). As shown in Table I, anionic liposomes caused a substantial decrease in effective inhibition by leupeptin, LBI, and SBI, as demonstrated by increases in the apparent \( K_d \) values. A 92-fold increase was observed with LBI, a 47-fold increase with leupeptin, and a 12-fold increase was observed with SBI.

A question arises as to whether the diminished inhibition of acrosin resulted from an effective lowering of the inhibitor concentrations through nonspecific absorption to the anionic liposomes. This possibility was examined through two approaches using the highly purified LBI, and in one case, leupeptin. First, the extent of enzyme inhibition over a 60-min incubation period was examined as a function of phospholipid concentration. As shown in Table II, a 10-fold increase in phospholipid did not significantly alter the inhibited initial velocities of either acrosin or a second proteinase, trypsin, when incubated with either LBI or leupeptin. Furthermore, the extent of trypsin inhibition was essentially unchanged either in the presence or absence of the liposomes. These data...

**Fig. 1 (left).** Progressive inhibition of liposome-bound and solubilized acrosin. Liposome-bound (solid points) and solubilized acrosin (open points) were incubated with leupeptin (●), LBI (○), or SBI (△) and assayed for enzyme esterase activity at the indicated intervals. Acrosin activity represents nanomoles of BzArgOEt min\(^{-1}\) ml\(^{-1}\). For details, see "Experimental Procedures."

**Fig. 2 (right).** Progressive inhibition of liposome-bound and solubilized acrosin by an endogenous inhibitor from sperm extracts. Acrosin (0.93 μg) was preincubated with or without (●) 0.3 μmol of phospholipid in 2.9 ml of buffer at 24 °C. After 5 min, a 0.1-ml aliquot of partially purified inhibitor was added and the mixtures were assayed at the indicated intervals. Acrosin activity represents nanomoles of BzArgOEt min\(^{-1}\) ml\(^{-1}\). For details, see "Experimental Procedures."
strongly suggest that there was no apparent loss of available inhibitor through absorption to the liposomes, since increasing the area of membrane surface did not result in loss of inhibition. This finding is further supported by a direct binding assay in which mixtures of LBI (0.5 mg/ml) and phospholipid (0.5 μmol/ml) were incubated for 60 min at pH 8.0, centrifuged at 160,000 × g for 30 min (17) and the resulting supernatant and pelleted phospholipid fractions subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (28). After staining for protein with Coomassie Brilliant Blue R-250, intensely stained bands corresponding to LBI (M, = 9000) were observed in the gels of the supernatant fractions, but no stained bands were observed in the phospholipid fractions (results not shown), indicating an absence of significant protein-liposome binding. Thus, the ability of anionic liposomes to reduce acrosin inhibition appears not to be a function of direct inhibitor-membrane binding interactions.

While anionic liposomes appear to diminish the inhibition of acrosin by progressive inhibitors, the liposomes did not have a significant effect on acrosin inhibition by benzamidine or p-aminobenzamidine, two readily reversible, active site-directed, competitive inhibitors. The dissociation constants (K) for both of these inhibitors were essentially unchanged, in comparing lipid-bound and -solubilized acrosin (Table III). Furthermore, it has previously been shown that under the same buffering conditions, lipid-bound and -solubilized acrosin were almost identical with respect to substrate (BzArgOEt) binding (K) and catalytic efficiency (Vmax) (17).

The mechanism by which anionic liposomes cause a reduction in progressive inhibition of acrosin is not fully apparent from the above data, but several possibilities may be considered. First, the proximity of the enzyme to the membrane surface might cause steric hindrance by physically blocking access to the active site. This possibility is unlikely since it has previously been shown (17) that high molecular weight protein substrates (azocasein and azoalbunin) are hydrolyzed to the same extent by both lipid-bound and -solubilized acrosin. Furthermore, if physical obstruction is a function of the ligand molecular weight, one would not expect to see a dramatic reduction in inhibition by leupeptin, which has a molecular weight of 495, in comparison with LBI and SBI which have respective molecular weights of approximately 9,000 and 22,500.

The association of acrosin with liposomes does not appear to significantly alter the catalytic site of the enzyme since both ester and protein substrates (17), as well as low molecular weight competitive inhibitors (Table III) appeared to interact with the solubilized and bound enzyme equally. Another possible mechanism for the reduced inhibition of liposome-associated acrosin by leupeptin, LBI, and SBI is that the phospholipids induce changes in the enzyme tertiary structure which do not affect the active site but do affect other portions of the protein, possibly involved in the functional binding of the progressive inhibitors. Clearly, further study will be required to determine the validity of this hypothesis.

The observed differences in membrane-associated and -solubilized acrosin may have physiological implications with respect to enzyme regulation by inhibitors. Little is currently known about endogenous acrosin inhibitors but the results presented in this communication and elsewhere (10) suggest that at least some of these inhibitors have a lower affinity for the membrane-bound enzyme. While liposomes are at best a crude simulation of natural membranes, these results are in accord with the in situ study of Brown and Hartree (10), but without the presence of unknown constituents. Finally, the differences in enzymatic properties of membrane-bound acrosin may be of particular importance in the design of specific synthetic inhibitors as probes of acrosin function and as possible fertility regulatory agents.

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