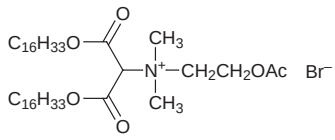
Vesicle–enzyme communication

Fredric M. Menger,* Kingsley H. Nelson and Yingbo Guo

Department of Chemistry, Emory University, Atlanta, Georgia, 30322, USA

Acetylcholinesterase and chymotrypsin are able to catalyze hydrolyses of vesicle-bound substrates at rates that depend upon the ability of the substrates to project beyond the membrane surface.

Pathogenic cells have been identified that produce specific enzymes in excessive amounts. For example, bone cancer has been shown to exude unusually large quantities of alkaline phosphatase,[1] while neuroblastomas generate high levels of acetylcholinesterase.[2] Earlier investigations from our laboratory have exploited this phenomenon in connection with a new and potentially specific mode of drug delivery involving compound **1**.[3] There were two key reasons for synthesizing **1**:



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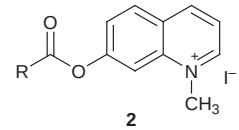
(i) It possesses a pair of hydrophobic tails plus a charged headgroup, the components essential for vesicle formation; (ii) It is a substrate for acetylcholinesterase, an enzyme that removes the acetate from the acetylcholine-like moiety. When enzymatic hydrolysis occurs on the 'vesiclized' compound, the freed hydroxy group reacts with one of the ester groups to form a six-membered lactone, thereby eliminating a hydrocarbon tail. But since amphiphiles with a single tail are generally unable to maintain a bilayer structure, the vesicle ruptures and releases its internal contents. The process is enzyme-specific, leading to the possibility of selective release near a cancer cell that overproduces the particular enzyme.

The preceding mechanism presupposes a reaction between a water-soluble enzyme and a vesicle-bound substrate. Somehow, in a manner not yet understood, the substrate within the bilayer membrane is able to find its way to the active site. It seems unlikely that, prior to enzyme binding, a substrate migrates *in toto* from the membrane into the aqueous domain. If this were possible, vesicles would lose and exchange their water-insoluble lipids rapidly, which they do not.[4] A reasonable alternative is that substrates only partially extend themselves beyond the membrane surface. Thus, a lipid molecule might transiently expose its polar moiety (and even portions of its hydrocarbon chains) to the bulk solvent. In this way an enzyme would have an opportunity to grasp the substrate.

An X-ray structure of acetylcholinesterase *(Torpedo californica)* shows that the active site (containing a Ser-His-Glu triad) lies at the bottom of a 'deep and narrow gorge'.[5] Hence, a membrane-bound substrate must seemingly endure a tortuous journey as it leaves the membrane and travels down the cleft to the active site. In actual fact, the journey may not be as difficult as it appears. The cleft is lined with aromatic amino acids that are believed to guide into the active site a substrate molecule reaching the outer rim of the cleft. If this is correct, then one could visualize the escorting of a substrate molecule, which happens to protrude from the membrane, to the active site as soon as a vesicle and a properly oriented enzyme come into contact. It was the purpose of the work reported herein to learn more about such enzyme–vesicle communication.

Substrates in our study consisted of acylated *N*-methyl-7-hydroxyquinolinium iodide derivatives **2**.[6] Acetylcholinester-



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ase-catalyzed ester hydrolysis leads to a highly fluorescent 7-hydroxyquinolinium salt (λ_{ex} = 400 and 500 nm), allowing kinetics to be carried out spectrofluorimetrically at substrate concentrations of only 5 μM (phosphate buffer; pH 6.0; 25.0 °C), a concentration far too low to cause membrane disruption. Substrates with hydrophobic R groups were incorporated into the vesicle bilayers during their formation. Vesicles, consisting of dilauroylphosphatidyl choline plus 5% dimyristoyl phosphatidic acid (to inhibit floculation),[7] were prepared with the aid of a LiposoFast low-pressure extruder equipped with a 0.1 μm polycarbonate filter.[8] Monodisperse unilamellar vesicles of about 100 nm diameter (dynamic light scattering) at a total lipid concentration of 1.7 mM were thereby achieved. The concentration of acetylcholinesterase (Sigma electric eel, type V-5) added to the vesicular substrates was about 0.7 μM as determined by enzyme titration.[9]

In the absence of vesicles, but otherwise under the conditions specified above, the acetyl form of **2** (called 'C_2') reacts instantly with acetylcholinesterase. Longer-tailed derivatives are slower: the octanoyl, dodecanoyl, and tetradecanoyl esters ('C_8', 'C_{12}' and 'C_{14}') have half-lives varying from 20–60 s. The hexadecanoyl ester ('C_{16}') is not completely hydrolyzed even after half an hour. Although micellization of C_{16} is not a factor in the slow rate (its concentration lies well below the critical micelle concentration), it is quite possible that chain-coiling, as discussed in another context,[10] sterically impedes access to the headgroup by the enzyme.

Introducing vesicles into the system had no effect upon the fluorescence *vs*. time plots for C_2 even at elevated phospholipid concentrations (Fig. 1). Apparently, the hydrophilic substrate fails to bind to the lipid bilayers. C_8 also displays little rate change when co-mixed with phospholipid, a result explainable by either an absence of vesicle binding or by an efficient enzyme catalysis on bound ester. NMR data, given below, strongly favor the latter. In contrast, vesicular substrates C_{10}–C_{16} experience substantial rate inhibitions, the magnitude of which depend on the length of the chain (Fig. 1). Thus, C_{10} has a half-life of 300 s, whereas C_{14} and C_{16} are, for all practical purposes, inert. The C_{14} and C_{16} tails likely serve to anchor the substrates, *i.e.* to impede the ability of the headgroups to project beyond the membrane surface where reactive encounters with the enzyme's cleft become possible. Note that the data in Fig. 1 have practical implications for prodrug design because release of a membrane-bound moiety depends critically upon the structure of the disposable addendum.[11]

NMR spectra in the absence and presence of vesicles leave no doubt that C_8 (and, by inference, all substrate with chains longer

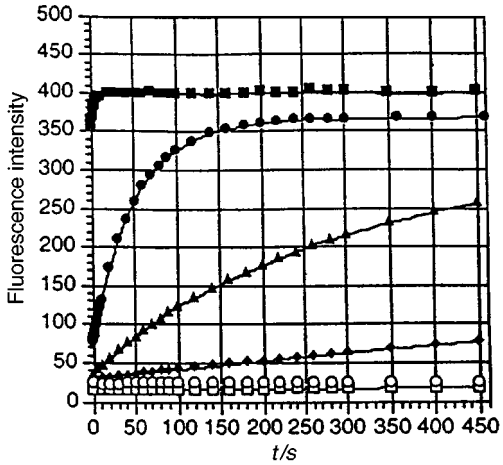


Fig. 1 Change in fluorescence intensity at 500 nm as a function of time in the acetylcholinesterase-catalyzed hydrolysis of vesicular substrates (■) C_2, (●) C_8, (▲) C_{10}, (◆) C_{12}, (□) C_{14} and (○) C_{16} under conditions specified in the text. Note how the reaction rate under standard conditions diminishes with the length of the acyl group.

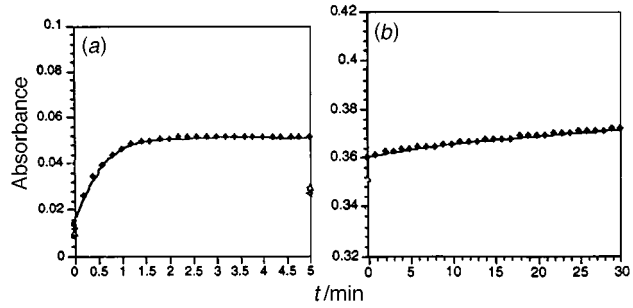
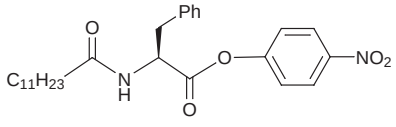


Fig. 2 Change in absorbance at 400 nm as a function of time in the α-chymotrypsin-catalyzed hydrolysis of **3** under conditions specified in the text. (*a*) with no vesicles and (*b*) with vesicles. Absorbance values are higher for the vesicular system owing to scattering by the vesicles.

than eight) is substantially vesicle-bound under the experimental conditions.[12] It was found that the sharp aromatic peaks in free solution are obliterated by the vesicles, an observation consistent with substrate immobilization due to bilayer interactions. As would be expected, hydrophilic C_2 was shown to preserve its sharp signals in the presence of vesicles. The results with C_8 prove that an enzyme can maintain a normal rate despite membrane adsorption of its substrate. In terms of our 'protrusion model', the octanoyl group, but not the more hydrophobic tetradecanoyl or hexadecanoyl group, permits escape of the headgroup from the membrane surface into the clutches of the enzyme. The term 'escape' could signify either a dynamic process (in which C_8 rapidly relocates itself in the presence of an enzyme) or a static process (in which C_8 exists in equilibrium among multiple sites regardless of enzyme).

Two additional experiments are relevant to the above conclusions. (i) Tripling the vesicle concentration at a constant 5 μM substrate had little impact on the kinetics. Total binding of the C_{12} and higher substrates is thus assured. (ii) Instead of mixing C_{10} with the phospholipid prior to vesicle formation, C_{10} was added after substrate-free vesicles had been extruded. Since no difference in the kinetics was observed, the substrate in solution must associate with the vesicles rapidly.

To obtain additional information on enzyme–vesicle interactions, we examined a second water-soluble enzyme, chymotrypsin, operating on substrates **3** and **4**. Fig. 2 compares the



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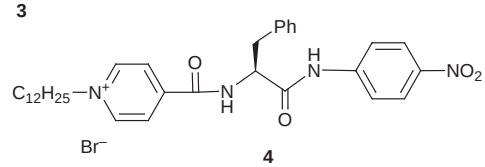

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hydrolysis of **3** in the absence of vesicles (phosphate buffer; pH 7.0; 25.0 °C; [substrate] = 5.5 μM; [enzyme] = 0.46 μM) with the hydrolysis of **3** in the presence of vesicles (5.3 mM total phospholipid). A two-order-of-magnitude inhibition by the vesicles is evident. With the goal of further modifying vesicular rates, the 'spacer' between the chain and ester group was extended *via* additional amino acids (*e.g.* dodecanoyl-Gly-Gly-Phe-COOAr) or *via* oxyethylene units (*e.g.* dodecyl-$(OCH_2CH_2)_8$OCO-Phe-COOAr).[13] Unfortunately, the non-vesicular rates were too slow to allow the dependency of rate on spacer length to be systematically examined. Compound **4** behaved well, however, and showed actually a 2.5-fold *faster* initial rate when incorporated into vesicles under our standard conditions. The cationic charge on the pyridinium ring likely positions the ester group into the water away from the membrane surface. Both electric charge (to prevent burying of the reactive headgroup) and spacer rigidity (to prevent looping back of headgroups onto the membrane surface) should favor enzyme–vesicle reactions. These features will be incorporated into future prodrug design.

Vesicle membranes clearly offer an attractive means of controlling enzymatic rates and specificities.

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Notes and References

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