Evidence for an Umbrella Mechanism of Bilayer Transport

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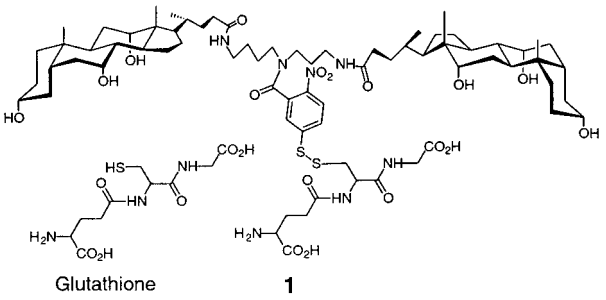
We have recently shown that a conjugate derived from cholic acid, spermidine, and Ellman's reagent, bearing covalently attached glutathione (i.e., **1**) readily crosses phospholipid bilayers.[1] Our working hypothesis has been that such transport occurs via an "umbrella" mechanism in which the conjugate traverses the membrane in a shielded conformation. A stylized illustration of the putative, transport-active species is shown in Chart 1A, where, each sterol appears as a doubly shaded rectangle having a hydrophobic (darkened) and a hydrophilic (lightly shaded) face; the lightly shaded oval corresponds to the hydrophilic peptide. In this paper, we present experimental support for such a mechanism.



The first question that we sought to answer was the molecularity of the transport process; that is, whether **1** crosses lipid bilayers by itself or in an aggregated form. As discussed previously, unambiguous evidence that **1** can enter a phospholipid vesicle, containing entrapped glutathione (GSH), can be obtained from: (i) the formation of oxidized glutathione (GSSG) within the interior of the vesicle, (ii) the appearance of the thiol form of the umbrella (USH), and (iii) the absence of release of GSH into the external aqueous phase; that is, a chemical reaction takes place within the vesicle interior according to eq 1

$$\mathbf{1} + \mathrm{GSH} \rightarrow \mathrm{USH} + \mathrm{GSSG} \qquad (1)$$

(see also Scheme 1).[1] Although large unilamellar vesicles (200 nm diameter) derived from 1-palmitoyl-2-oleyol-*sn*-glycero-3-phosphocholine (POPC) can be used to demonstrate the ability of **1** to cross lipid bilayers, the fact that the rate-limiting step in this system is a chemical reaction precludes its use for studying the transport process, directly.[1] Recently, we reasoned that diffusion through thicker bilayers might shift the rate-limiting step toward membrane permeation. To our satisfaction, we have found this to be the case, using bilayers made from [1,2-di(13-*cis*-docosenoyl)-*sn*-glycero-3-phosphocholine] (**C**22:1) plus 5% 1-palmitoyl-2-oleyol-*sn*-glycero-3-phosphatidyl glycerol (POPG). Thus, addition of submicellar concentrations of **1** (15 μM) to large unilamellar vesicles (200 nm diameter) formed from this lipid mixture, which contained 2.0 mM of entrapped GSH, resulted in

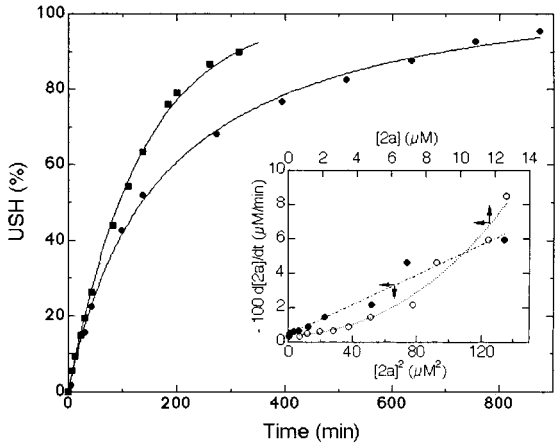
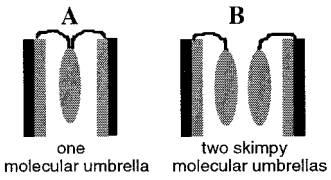
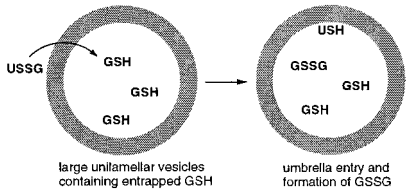


Figure 1. Appearance of USH for the reaction of 0.2-μm (**C**22:1)/POPG (95/5) liposomes (11.1 mM) containing 2.0 mM GSH with 15 μM of **1** (■) and **2a** (●) at 40 °C. The solid lines represent theoretical curves for first-order and second-order processes, respectively; inset shows plots of rate as a function of the remaining concentration of **2a** to the second power (●, lower *x*-axis) and to the first power (○, upper *x*-axis).

Chart 1



Scheme 1



complete reaction at 40 °C without leakage of GSH. Increasing the internal GSH concentration to 10 mM resulted in a negligible increase in rate, indicating that membrane permeation is rate-limiting. Analysis of these rate data gave a good fit to first-order kinetics, further indicating the involvement of transport-active *monomers* (Figure 1). Specific procedures that were used to carry out these experiments were similar to those previously described.[1−6]

A second question that we wanted to address in this study was the minimum number of facially amphiphilic units needed for membrane transport.[3] Our presumption has been that at least *two* such units are necessary to provide sufficient coverage for masking the hydrophilicity of the attached peptide. In principle, however, one can imagine that an analogue of **1**, bearing a single facially amphiphilic unit (i.e., a "skimpy" molecular umbrella), might

(1) (a) Janout, V.; DiGiorgio, C.; Regen, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2671. (b) Janout, V.; Zhang, L. H.; Staina, I. V.; DiGiorgio, C.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 5401.

(2) Borate buffer that was used in all of the experiments described in this work was composed of 0.1 M H_3BO_3, which contained 2 mM EDTA, and was adjusted to pH 7.0 by titration with 1 M NaOH.

(3) For a discussion of facial amphiphilicity, see: (a) McQuade, D. T.; Barrett, D. G.; Desper, J. M.; Hayashi, R. K.; Gellman, S. H. *J. Am. Chem. Soc.* **1995**, *117*, 4862. (b) Venkatesan, P.; Cheng, Y.; Kahne, D. *J. Am. Chem. Soc.* **1994**, *116*, 6955.

(4) Janout, V.; Lanier, M.; Regen, S. L. *J. Am. Chem. Soc.* **1996**, *118*, 1573.

(5) Janout, V.; Lanier, M.; Regen, S. L. *J. Am. Chem. Soc.* **1997**, *119*, 640.

(6) Shawaphun, S.; Janout, V.; Regen, S. L. *J. Am. Chem. Soc.* **1999**, *121*, 5860.

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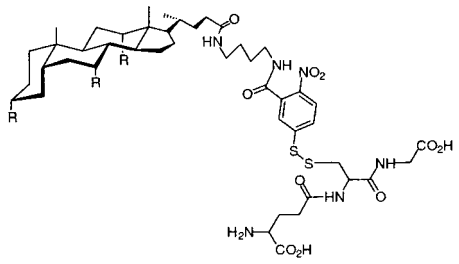
cross a lipid bilayer, but only in an aggregated form. For example, a noncovalent dimer (two skimpy umbrellas arranged in a shielded conformation) would provide coverage that approaches that of one molecular umbrella bearing two facially amphiphlic units (Chart 1B).

To probe this question, conjugate **2a** was synthesized and its transport properties investigated. Thus, monoacylation of putrescine with cholic acid, followed by condensation with an activated form of Ellman's reagent produced the corresponding symmetrical disulfide.[1] Subsequent reaction with GSH afforded **2a** having λ_{max} 330 nm (ϵ 5500 M^{-1} cm^{-1}, borate buffer, pH 7.0). The corresponding thiol monomer, generated via the reduction of **2a** with tris(2-carboxyethyl)phosphine (TCEP), exhibited a λ_{max} 427 nm (ϵ 4000 M^{-1} cm^{-1}, borate buffer) and a critical micelle concentration of 120 μM (surface tension). Using experimental procedures similar to those used for **1**, an excess of vesicles of (**C**22:1)/POPG (95/5, mol/mol), which had been loaded with 2.0 mM GSH, was exposed to a submicellar concentration of **2a** at 40 °C. Complete reaction was observed, which was characterized by a *second-order* dependency on the umbrella concentration (Figure 1). Control experiments established that GSH did not leak out of the vesicles during the reaction and that GSSG was formed within the aqueous interior. A similar experiment that was carried out, in which the concentration of entrapped GSH was increased to 10.0 mM, showed, essentially, the same rate of appearance of USH. These results indicate that membrane permeation is rate-limiting and that *dimers* of **2a** are the transport-active species.



2a, R=OH
b, R=H

The third, and most important, issue that we sought to clarify was whether the ability of molecular umbrellas to promote the transport of glutathione across lipid bilayers was simply the result of a shift in its hydrophobic/hydrophilic balance.[7−9] To examine this question, an analogue of **2a** (i.e., **2b**) was synthesized that was devoid of sterol hydroxyl groups. Thus, if an umbrella mechanism were operative, **2a** (but not **2b**) should be able to cross lipid bilayers. Conversely, if enhanced permeability of the glutathione molecule were due to an overall increase in hydrophobicity via conjugation with the sterol, then **2b** should cross bilayers more readily than **2a** since it is more hydrophobic. Conjugate (**2b**), synthesized from the methyl ester of cholanic acid, showed UV characteristics that were similar to those of **2a**: its critical micelle concentration of 32 μM.[10] As expected, **2a** was found to be less mobile than **2b** on silica gel due to its greater hydrophilic character; that is, the R_f values for **2a** and **2b**, using $CHCl_3/CH_3OH/H_2O$ (60/40/10, v/v/v) as an eluting solvent, were 0.34 and 0.62, respectively.

To maximize the rate of membrane transport by **2a** and **2b**, thin POPC vesicles were chosen as targets. When POPC vesicles (loaded with 1.2 mM GSH) were exposed to a submicellar concentration of **2a** at 23 °C, a complete reaction was observed, which was characterized by pseudo-first-order kinetics and a half-life of 38 min. A similar experiment that was carried out, in which the concentration of GSH was increased to 2.0 mM, resulted in a proportional increase in the observed pseudo-first-order rate constant; that is, the half-life was reduced to 23 min, indicating that the chemical reaction is rate-limiting. Control experiments established that GSH did not leak out of the vesicles during the reaction and that GSSG was formed within the vesicles. In sharp contrast, an analogous experiment that was carried out with **2b** showed *negligible reaction after 18 h*. In separate experiments, incubation of multilamellar vesicles of POPC with **2a** (and also with **2b**), followed by centrifugation and analysis of the supernatant, showed that both conjugates were almost fully bound (>95%) to the membrane.

Two explanations that can account for the absence of reaction of **2b** with vesicle-entrapped GSH are that its rate of transbilayer movement is very slow or its rate of reaction with GSH at the membrane−water interface is negligible. To judge the reactivity of membrane-bound **2b**, we carried out a "double-sided" experiment in which the conjugate was included as a membrane component during vesicle formation.[11] In principle, if transbilayer movement is very slow and chemical reaction is relatively fast, then addition of GSH to the external aqueous phase should result in ca. 50% of the conjugate undergoing reaction. Using concentrations of vesicles, GSH, and **2b**, which were similar to those used in the above single-sided addition, we found that 55% of the conjugate underwent reduction with a half-life of 11 min, and that ca. 45% of **2b** was unreactive. The full extent of the reaction was quantified by (i) removing external GSH via gel filtration (Sephadex G-25), after the dispersion was allowed to incubate for 2.5 h, (ii) dissolving the vesicles with an excess of sodium dodecyl sulfate, and (iii) analyzing the solution before and after treatment with excess dithiothreitol. The observation that only about half of **2b** was reactive toward externally added GSH confirms that the conjugate crosses lipid bilayers very slowly, if at all. Taken together, these results rule out the possibility that the greater membrane permeability found with **2a** is a consequence of its hydrophobic/hydrophilic balance. Instead, they provide compelling evidence for an umbrella mechanism of transport.

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Supporting Information Available: Procedures for the synthesis of **2a** and **2b**, and Figure showing the kinetics of **2a** reacting with GSH entrapped in POPC liposomes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA016265A

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(9) In preliminary studies, we have found that a deoxycholic acid-based analogue of **2a** also readily crosses POPC membranes.

(10) For **2b**, λ_{max} 333 nm (ϵ 3360 M^{-1} cm^{-1}, borate buffer) and λ_{max} 420 nm (ϵ 6100 M^{-1} cm^{-1}, borate buffer).

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