

Environmental Effects Dominate the Folding of Oligocholates in Solution, Surfactant Micelles, and Lipid Membranes

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Abstract: Oligocholate foldamers with different numbers and locations of guanidinium–carboxylate salt bridges were synthesized. The salt bridges were introduced by incorporating arginine and glutamic acid residues into the foldamer sequence. The conformations of these foldamers were studied by fluorescence spectroscopy in homogeneous solution, anionic and nonionic micelles, and lipid bilayers. Environmental effects instead of inherent foldability were found to dominate the folding. As different noncovalent forces become involved in the conformations of the molecules, the best folder in one environment could turn into the worst in another. Preferential solvation was the main driving force for the folding of oligocholates in solution. The molecules behaved very differently in micelles and lipid bilayers, with the most critical factors controlling the folding–unfolding equilibrium being the solvation of ionic groups and the abilities of the surfactants/lipids to compete for the salt bridge. Because of their ability to fold into helices with a nonpolar exterior and a polar interior, the oligocholates could transport large hydrophilic molecules such as carboxyfluorescein across lipid bilayers. Both the conformational properties of the oligocholates and their binding with the guest were important to the transport efficiency.

Introduction

Environments can have strong influences on the conformation of a molecule. Proteins, for example, frequently change their conformation as they bind to ligands, come in contact with membranes, or interact with other proteins. As the environment of a molecule becomes different, the molecule experiences different noncovalent interactions with the surrounding and, in turn, a different balance of intra- and intermolecular forces crucial to its conformation. The strong impact of environments on biomolecules already made some researchers wonder how relevant the learning from conformational studies of proteins in test tubes is to what the molecules do in natural settings.¹ Unlike a dilute solution, some intracellular compartments have macromolecules reaching 500 g/L in concentration. The conformation of proteins and other macromolecules sometimes differs greatly in these crowded environments from those in dilute, homogeneous solution. Human amyloidogenic proteins related to neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, for example, were found to be strongly affected by cell-like crowded environments.²

Intrigued by the exquisite conformational control of biomolecules by nature, chemists began to design and synthesize synthetic molecules (i.e., foldamers) adopting ordered, controllable conformations.³ The field of foldamers has undergone rapid development in recent years. As new folding motifs⁴ and building blocks⁵ were created, novel properties and functions, sometimes unavailable from biofoldamers, began to emerge. Naturally, the learning generated from biomimetic, synthetic foldamers was used to understand and solve biological problems. Foldamer-based synthetic antimicrobial agents,⁶ protein surface binders and inhibitors,⁷ vesicles and organogellators,⁸ and biomimetic enantioselective catalysts⁹ all appeared in the literature.

Despite the impressive progress made in synthetic foldamers, the research so far has been focused almost exclusively in homogeneous solution and in the solid state. Although some foldamers were prepared to interact with lipid bilayers,⁶ their

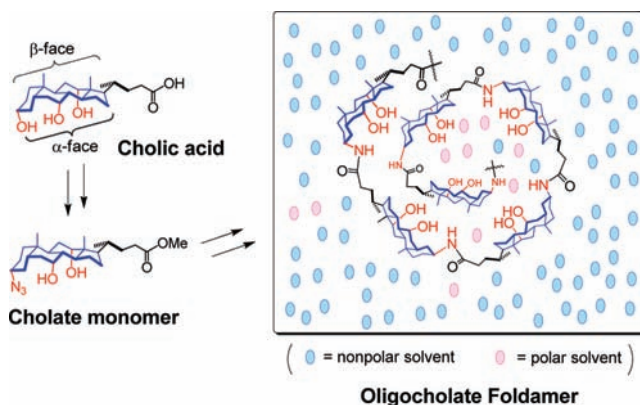
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conformations within membranes generally were not studied in detail. Lipid membranes are one of the most important environments for biofoldamers. Membrane proteins perform vital biological functions, such as photosynthesis, ion conduction, signal transduction, and immune response, and account for nearly 50% of all drug targets¹⁰ but are notoriously difficult to study. Biochemists have studied membrane proteins in both lipid bilayers and surfactant micelles.¹¹ Being amphiphilic in nature, the latter are considered good mimics of the bilayer environment and have yielded much insight into how membrane-associated proteins or peptides might fold in a membrane-like environment.

In this paper, we report the conformational study of several functionalized oligocholate foldamers in homogeneous solution, ionic and nonionic micelles, and lipid bilayers. To the best of our knowledge, no study has been reported to systematically investigate how these different environments influence the conformation of synthetic foldamers. We found that environmental effects can completely overwhelm the inherent foldability of a foldamer. Micelles are frequently used as a membrane-like environment to investigate the conformation of membrane-associated proteins/peptides, but our work indicates that the conformation of the same foldamers may differ greatly in micelles and lipid bilayers, or even in different micelles. One of the most difficult challenges in modern bioorganic chemistry is selective translocation of hydrophilic molecules across lipid bilayers. We demonstrate that the oligocholate foldamers are effective molecular transporters in common phospholipid membranes. The ability to correlate structure and activity and the straightforward functionalization of the foldamers are important

Scheme 1. Schematic Representation of the Solvophobic Folding of an Oligocholate in a Mixture of Polar and Nonpolar Solvents



to the rational design of molecular transporters useful in applications such as membrane separation and drug delivery.

Results and Discussion

Design and Synthesis of Oligocholates. An oligocholate folds best in nonpolar solvents containing a small amount of a polar solvent.¹² The polar solvent is needed not only to dissolve the oligomer but, more importantly, to provide the solvophobic driving force to the folding (Scheme 1). When the oligocholate folds into a helix, the rigidity of the steroidal backbone keeps the internal cavity from collapsing. Polar solvent is microphase-separated from the bulk solvent mixture and concentrated into the nanometer-sized hydrophilic cavity. Such an arrangement is favorable for two main reasons. First, the hydrophilic amide and hydroxyl groups of the oligocholate are efficiently solvated by the entrapped polar solvent in a largely nonpolar medium. Second, the phase-separated polar solvent can reside in a preferred, polar microenvironment instead of in the bulk, nonpolar solvent. A unique feature of this folding mechanism is its strong dependence on both the *size* and the *shape* of the polar and nonpolar solvents, derived partly from the dimension of a typical solvent molecule being significant in comparison to the size of the hydrophilic cavity.¹³

Chart 1 shows the structures of the oligocholates used in the current study. Because the cholate units are connected by amide linkages, we can introduce intramolecular salt bridges conveniently by incorporating arginine and glutamic acid residues in the sequence. The syntheses of the molecules followed previously reported procedures^{12–14} and are detailed in the Supporting Information. All oligocholates are labeled with a dansyl and a naphthyl group at the chain ends, which are used as the acceptor (A) and donor (D) for fluorescent resonance energy transfer (FRET).¹⁵ In FRET, the energy-transfer efficiency (E) is related to the D–A distance (r) by equation $E = R_0^6 / (R_0^6 + r^6)$, in which R_0 is the Förster distance for a specific D–A pair. Because typical R_0 (2–6 nm) is comparable to the diameter of a protein, FRET is widely used in the conformational

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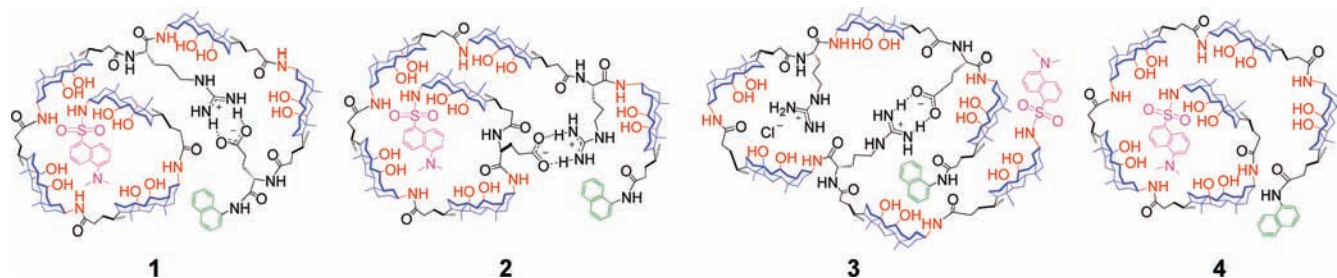
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Chart 1



study of biomolecules.¹⁵ Distance-dependent techniques such as NOE, FRET, and spin coupling¹⁶ are extremely powerful in the characterization of synthetic and natural foldamers.³ FRET has the benefit of measuring much larger distances than NOE and spin coupling and was found to work extremely well for the oligocholates.^{12–14}

Oligocholates **1** and **2** both contain a salt bridge; their difference lies in the number of cholate units in between the arginine and glutamic acid. Three cholates make one turn in the helix in the folded oligocholates;¹² thus, the absolute distance between the arginine and glutamic acid in the *folded* conformer is quite similar in **1** and **2**. Nonetheless, the salt bridge will be formed within the first helix turn in **1** and across two turns in **2**. If one thinks forming a salt bridge over a longer distance more effectively brings the chain to the folded conformation (as it impacts a larger part of the chain), **2** should fold better than **1**. If, however, one considers that formation of the salt bridge over a longer distance needs to constrain a larger section of the foldamer chain—an entropically unfavorable process—**2** might fold worse than **1**. Which is true?

Oligocholate **3** contains two arginines and one glutamic acid and thus carries a net positive charge. Examination of the CPK model suggests that the farther arginine is more likely to be engaged in the salt bridge, as ion-pairing over the large and rigid steroid backbone of the cholate may introduce significant strain to the structure. Will the extra arginine impact the conformation of the molecule? Compound **3** is the only one with a net charge. Many proteins are charged.¹⁷ Electrostatic interactions operate over much longer distances than most other noncovalent forces. How will the charge impact the conformation of the molecule? Will the impact be similar across different environments? How will the non-participating arginine affect its ability to protect a hydrophilic guest and transport it across lipid membranes?

As a control, **4** is the only foldamer made entirely of cholates (other than the fluorophores). Without any salt bridge, its inherent foldability should be the lowest among the four. Will it be the worst folder always?

Folding of the Oligocholates in Homogeneous Solution. To answer the above questions, we first studied the molecules in 2:1 hexane/ethyl acetate with different amounts of methanol, one of the most “folding-friendly” solvent systems identified for the oligocholates.¹³ Methanol is completely miscible with ethyl acetate but nearly immiscible with hexane. A high percentage of hexane reduces the energetic cost in the phase separation of methanol from the bulk to the hydrophilic cavity of folded helix (Scheme 1) and therefore is beneficial to the folding.

Because the same D–A pair is used in **1–4**, the energy-transfer efficiency directly reflects the distance between the donor and the acceptor. FRET may be determined either by the enhancement of the acceptor emission or by the weakening of the donor emission.¹⁵ In our experience, the naphthyl emission is weak in most solvents used to fold the oligocholates, and FRET is best determined by monitoring the acceptor emission. However, because dansyl itself is sensitive to solvent polarity¹⁸ and folding of an oligocholate changes the solvent composition near the fluorophore (Scheme 1), we cannot use the emission intensity directly to measure the energy-transfer efficiency. Instead, we need to extract the contribution of FRET (to the acceptor emission) from the excitation spectrum, obtained when the acceptor emission at 500 nm was monitored while the excitation wavelength (λ_{ex}) was scanned.¹⁹ In the absence of energy transfer, the excitation spectrum of a compound resembles the absorption spectrum of the acceptor. Energy transfer from the donor is indicated by peaks corresponding to the donor’s absorption.¹⁵

The excitation spectra of the oligocholates differ greatly. FRET is weak in **1** (Figure 1a) but very strong in **2** (Figure 1b) in low methanol solutions, evidenced by the large peak at 300 nm from the naphthyl donor in the latter. Location of the salt bridge thus has a strong effect on the energy transfer. The contribution from the donor gradually disappears as methanol is added. Toward the end of solvent titration, the spectra of **1** and **2** look identical, only showing the dansyl absorption near 260 and 340 nm.

Despite the large difference in structure, **3** and **4** have very similar excitation spectra at the beginning of the solvent titration with 2% methanol (Figure 1c,d). The difference between the two lies in their response to methanol. Whereas the donor absorption at 300 nm in **3** is slowly weakened upon methanol addition, it quickly disappears in **4** with as little as 6% methanol.

Before we can correlate the FRET data with the folding, we have to exclude intermolecular aggregation under the experimental conditions, as FRET simply reflects a close D–A distance and can come from either an intramolecular or an intermolecular process. Our previous work indicates that non-ionic oligocholates such as **4** do not aggregate under similar conditions.¹² Oligocholates **1–3**, however, contain highly polar, ionic groups. Figure 2a shows the excitation spectra of **3** in 2:1 hexane/ethyl acetate with 2% methanol over 0.13–2.0 μM . This is the most stringent test for aggregation, using the most polar oligocholate in the least polar solvent mixture. Nevertheless, the shape of the spectrum stays unchanged during the 16-fold dilution, and the normalized excitation spectra completely overlap with one another (Figure 1S, Supporting Information).

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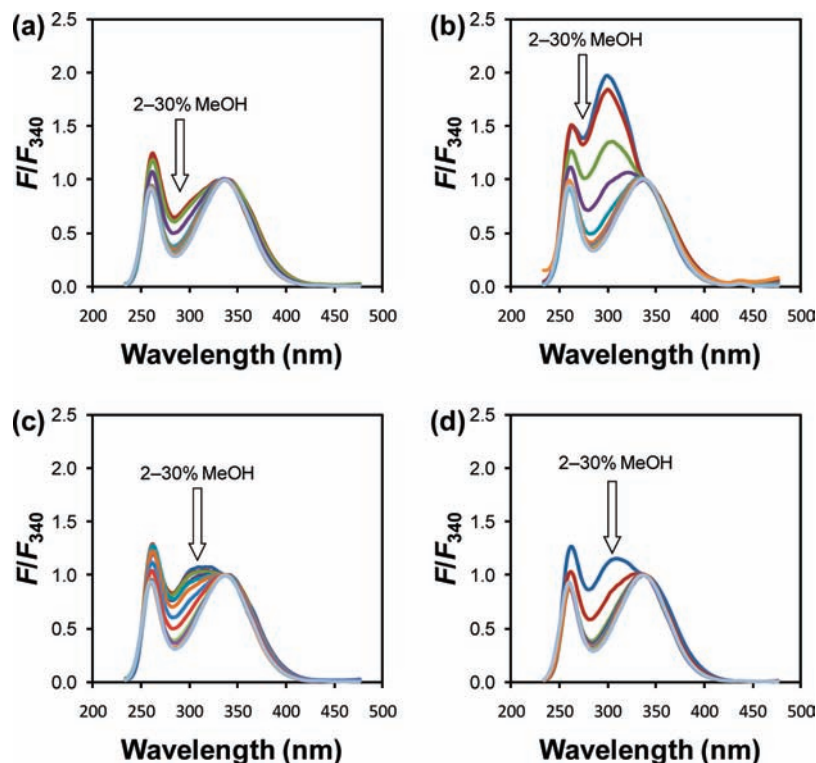


Figure 1. Normalized excitation spectra of (a) **1**, (b) **2**, (c) **3**, and (d) **4** in 2–30% MeOH in 2:1 hexane/ethyl acetate; [oligomer] = 2.0 μM . The acceptor emission at 500 nm was monitored. The intensity at 340 nm (the λ_{max} of dansyl in the absorption spectrum) was set to 1.

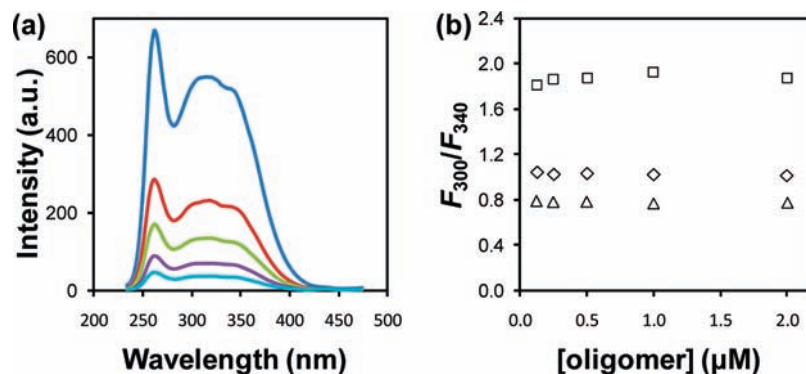


Figure 2. (a) Excitation spectra of **3** in 2% MeOH in 2:1 hexane/ethyl acetate. The concentration of **3** was 2.0, 1.0, 0.5, 0.25, and 0.13 μM from top to bottom. (b) F_{300}/F_{340} of **1** (Δ), **2** (\square), and **3** (\diamond) as a function of the oligocholate concentration. The acceptor emission at 500 nm was monitored. F_{300} and F_{340} represent the emissive intensity of dansyl at 500 nm in the excitation spectrum when λ_{ex} was 300 and 340 nm, respectively.

Thus, the FRET observed for the compound is independent of concentration, strongly supporting its intramolecular origin. The same is true for compounds **1** and **2** (Figures 2S and 3S, Supporting Information). Because the maximum absorption of naphthyl and dansyl occurs at 300 and 340 nm, respectively, we can use F_{300}/F_{340} in the excitation spectrum as an indicator for the FRET. A higher F_{300}/F_{340} is equivalent to a larger contribution from the donor and translates to a shorter D–A distance. As shown by Figure 2b, F_{300}/F_{340} is completely independent of oligomer concentration, suggesting that FRET comes from folding instead of aggregation in all these salt-bridged oligocholates.

Efficient FRET itself does not mean a compound is a good folder. The information about the foldability of a compound may be extracted from the change of FRET in response to solvent composition. This is very similar to the solvent-dena-

turation experiments for proteins²⁰ and is often described by a two-state model (eq 1).



In a two-state transition, only the fully folded and fully unfolded states exist at any given solvent composition. Such a conformational change is characterized by a sigmoidal titration curve. As shown by Figure 3a, the F_{300}/F_{340} curves for **1–4** are all sigmoidal in shape and fit well to the two-state model. (Details of the data analysis are given in the Supporting Information.) The data fitting allows us to calculate the folding equilibrium

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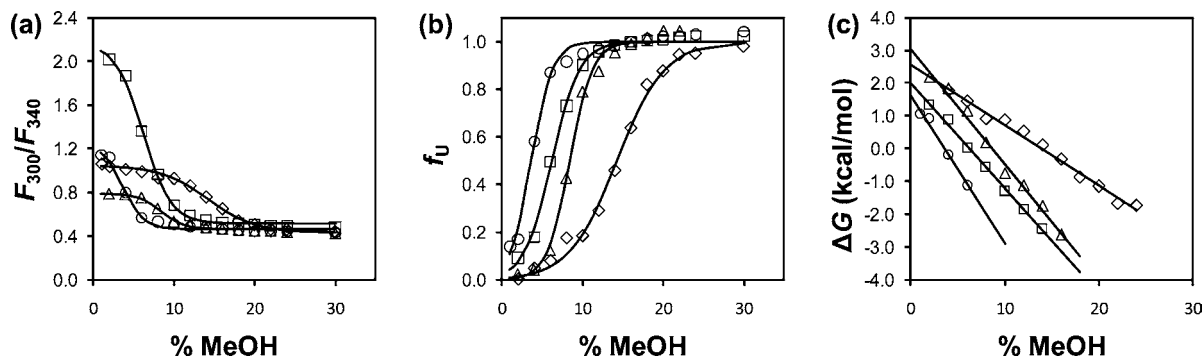


Figure 3. (a) F_{300}/F_{340} , (b) fraction of the unfolded conformer, and (c) unfolding free energies for **1** (Δ), **2** (\square), **3** (\diamond), and **4** (\circ) as a function of volume percentage of methanol in 2:1 hexane/ethyl acetate. The theoretical curves are nonlinear least-squares fitting to a two-state transition model in (a) and (b). Details for the curve-fitting are found in the Supporting Information; [oligomer] = 2.0 μ M.

Table 1. Values of ΔG_0 and m Determined from Solvent Denaturation Curves^a

entry	compound	solvent composition	ΔG_0 (kcal/mol)	m (kcal/mol)
1	1	MeOH in 2:1 hexane/ethyl acetate	3.5 ± 0.5 (3.1)	0.42 ± 0.06 (0.35)
2	2	MeOH in 2:1 hexane/ethyl acetate	2.1 ± 0.2 (2.0)	0.34 ± 0.03 (0.32)
3	3	MeOH in 2:1 hexane/ethyl acetate	2.8 ± 0.3 (2.6)	0.20 ± 0.02 (0.18)
4	4	MeOH in 2:1 hexane/ethyl acetate	1.7 ± 0.5 (1.7)	0.45 ± 0.09 (0.45)
5	1	MeOH in ethyl acetate	2.3 ± 0.4 (2.1)	0.40 ± 0.05 (0.36)
6	2	MeOH in ethyl acetate	0.6 ± 0.5 (0.5)	0.29 ± 0.05 (0.26)
7	3	MeOH in ethyl acetate	3.0 ± 0.4 (2.5)	0.19 ± 0.02 (0.16)
8	4	MeOH in ethyl acetate	— ^b	— ^b

^a ΔG_0 is the unfolding free energy of the compound in the absence of the methanol, and m represents the sensitivity of the unfolding free energy to the concentration of methanol in percentages. Data with errors are determined by nonlinear least-squares fitting to a two-state transition model. Data in parentheses are determined by linear fitting of the unfolding free energies as a function of denaturant concentration. See the Supporting Information for details. ^b Compound **4** was completely unfolded in methanol/ethyl acetate mixtures, and the unfolding free energies cannot be determined.

as a function of methanol percentage (Figure 3b). Another hallmark of the two-state transition is a linear dependence of free energies on the denaturant concentration, i.e., $\Delta G = \Delta G_0 - m[\text{MeOH}]$, where ΔG_0 is the unfolding free energy of the compound in the absence of the denaturant and m represents the sensitivity of the free energy to the concentration of the denaturant. As shown by Figure 3c, all four compounds display such a linear relationship.

The stability of the folded conformer is reflected by the amount of denaturant needed to induce 50% unfolding. The value is 8.4, 6.2, 14.3, and 3.7% methanol for oligocholates **1–4**, respectively (Figure 3b). Thus, although **2** has by far the most efficient FRET among the four, it is actually less stable than either **1** or **3**. Clearly, the location of the salt bridge impacts the folding strongly. The non-participating arginine must be important as well, since **3** has the same number of salt bridges as **1** and **2** but folds much better.

Table 1 summarizes the thermodynamic folding data obtained from the curving-fitting. These numbers provide a quantitative picture of how the folding–unfolding equilibrium is affected by the solvent composition. For the oligocholates, methanol plays dual roles in the folding–unfolding equilibrium. On one hand, the polar solvent is needed to dissolve the oligocholate and provide the preferential solvation of the hydrophilic faces of the cholates. On the other hand, when located outside the hydrophilic cavity and, in particular, present at higher concentrations, methanol can unfold or “denature” the helix. Thus, ΔG_0 is the unfolding free energy of the oligocholate predicted by the preferential solvation model extrapolated to 0% methanol. The number is purely theoretical in nature because the presence of methanol is necessary for the preferential solvation to operate.

As expected, without any salt bridge, **4** has the lowest ΔG_0 of 1.7 kcal/mol (Table 1, entry 4). It is somewhat surprising

that the guanidinium–carboxylate salt bridge does not bring much stabilization to **2**, as its ΔG_0 is only 0.3–0.4 kcal/mol larger than that of **4**. The small increase of ΔG_0 does not mean that the salt bridge is weak in the folded **2**. The association constant between guanidinium and carboxylate in hexane/ethyl acetate is unknown (and cannot be determined because of insolubility) but must be extremely large in such a nonpolar solvent mixture.²¹ If one assumes that the salt bridge is formed in both the folded and unfolded states, a small contribution to ΔG_0 simply means that the salt bridge does not help the folded state relative to the unfolded. There are four cholate groups between the salt bridge and multiple flexible methylene groups connecting the guanidinium and carboxylate to the foldamer backbone in **2**. If the salt bridge can be accommodated equally well by the folded and unfolded conformers, its formation will not make much difference to the folding–unfolding equilibrium; this is likely to be the case when the salt bridge is separated by a long, relatively flexible segment of a chain. If, however, the salt bridge is favored by either the folded or unfolded conformer, whether due to geometrical constraint or something else, its formation will help shift the equilibrium to that particular conformer.

Consistent with the above explanation, the unfolding free energy increases monotonically as the number of cholate units is reduced between the arginine and glutamic acid. For **3** and **1**, whose salt bridge is separated by three and two cholates, $\Delta G_0 = 2.8$ and 3.5 kcal/mol, respectively. It appears that, the closer the salt bridge, the more it can constrain the chain to the folded conformation and the less able is the chain to adopt an

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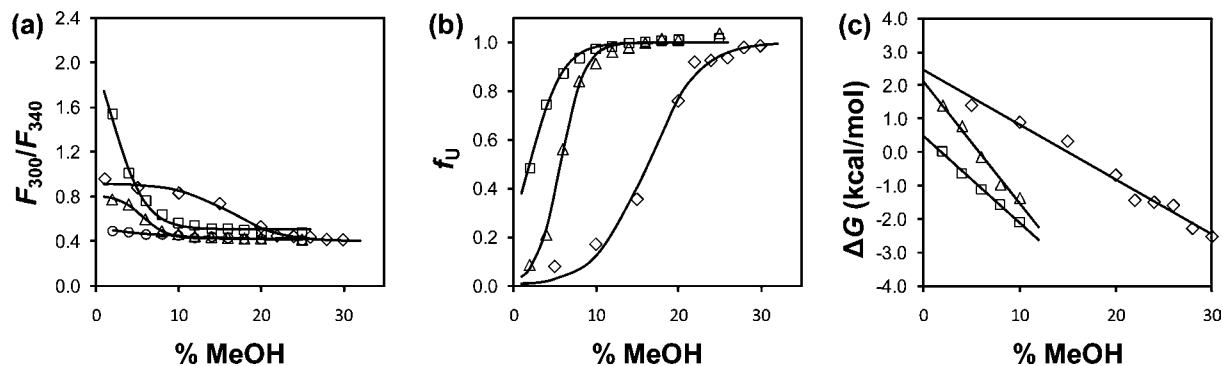


Figure 4. (a) F_{300}/F_{340} , (b) fraction of the unfolded conformer, and (c) unfolding free energies for **1** (Δ), **2** (\square), **3** (\diamond), and **4** (\circ) as a function of volume percentage of methanol in ethyl acetate. The theoretical curves are nonlinear least-squares fits to a two-state transition model in (a) and (b). [Oligomer] = $2.0 \mu\text{M}$.

extended conformation. The largest difference in ΔG_0 in these oligocholates is between **1** and **4**, amounting to 1.4–1.8 kcal/mol, depending on the (linear or nonlinear) method of curving-fitting.

Parameter m indicates how sensitive the unfolding reaction is to methanol percentage. The most sensitive foldamer among the four is nonionic **4**, with each percent methanol causing 0.45 kcal/mol of change in the unfolding free energy (Table 1, entry 4). The salt bridge does make the foldamer more resistant to methanol denaturation. The difference between **1** and **2** is not large, but m is slightly larger for **1** according to both linear and nonlinear curving-fitting. The trend is conceptually reasonable, as an increase in methanol not only could diminish the preferential solvation responsible for folding the oligocholate but also weaken the salt bridge. The more the folded conformer benefits from the salt bridge (in ΔG_0), the more sensitive the folding/unfolding equilibrium should be toward methanol.

Foldamer **3** is the least sensitive to methanol among the four. Hence, the non-participating arginine is quite important to the folding equilibrium. According to the preferential solvation model (Scheme 1), the concentration of methanol in the entire mixture is highest inside the folded helix. We previously utilized this nanosized pool of polar solvent for size-selective catalysis.²² This local methanol undoubtedly can weaken the hydrogen-bonded salt bridge but, in the meantime, can better solvate the non-participating guanidinium group, a highly favorable process in the largely nonpolar mixture. Thus, although the extra arginine does not contribute directly to the inherent foldability of the oligocholate (compare ΔG_0 of **1** and **3**, for example), it dampens the adverse impact of methanol by exploiting the localized methanol in the folded conformer.

To further confirm the above conclusions, we performed the solvent titration in methanol/ethyl acetate mixtures. Without hexane, folding is expected to be more difficult due to the higher cost in phase-separating methanol. Indeed, the non-salt-bridged **4** completely loses its ability to fold in the binary mixture (Figure 4a). The data for **1–3** continue to fit well to the two-state model, yielding the folding equilibria (Figure 4b) and unfolding free energies (Figure 4c). Interestingly, the ΔG_0 for **1** and **2** is decreased by 1–1.5 kcal/mol by eliminating hexane, while m is hardly affected (Table 1, compare entries 5 and 6 with entries 1 and 2). It seems that solvent miscibility, which is the core of the preferential solvation model, is more important to the inherent foldability. In a more challenging solvent, the difference

in m (0.40 vs 0.29) is more obvious for **1** and **2** than in the ternary solvents (0.42 vs 0.34). The data once again support that methanol is more detrimental to the folded conformer that benefits more from the salt bridge. On the other hand, the solvent change makes no difference to oligocholate **3**—its ΔG_0 and m are essentially the same with and without hexane. Thus, the extra arginine makes the foldamer more resistant to adverse solvent conditions, whether the addition of methanol or the elimination of hexane.

Folding of the Oligocholates in Surfactant Micelles. Dominated by hydrophobic groups, the oligocholates can be solubilized by surfactant micelles in water. Parent oligocholates such as **4** were found to fold well in sodium dodecyl sulfate (SDS) micelles.²³ The folding, however, follows a mechanism completely different from that in solution. The SDS micelle (ca. 3 nm in diameter) can accommodate the folded conformer much better than the unfolded. Each cholate unit is 1.4 nm from head to tail. According to CPK models, a fully folded cholate hexamer is less than 2 nm in dimension, but an unfolded conformer can stretch to several nanometers in length, depending on the exact conformation. Because the SDS micelles prefer to maintain the spherical shape in water, solubilizing an oligocholate within the micelle is analogous to pushing a snake into a small cage—the result is that the snake (oligocholate) has no choice but to coil up (fold).

How will the salt bridge and charge impact the folding in micelles? Once again, we recorded the excitation spectra of these compounds in 1–70 mM SDS. The F_{300}/F_{340} value is 0.75, 1.54, and 1.14 for compounds **1–3**, respectively, in 1 mM SDS. These numbers match quite well with those of the corresponding folded conformers ($F_{300}/F_{340} = 0.78, 2.01, \text{ and } 1.04$) in 2% methanol in 2:1 hexane/ethyl acetate, suggesting that the oligocholates are probably folded in low SDS solutions (vide infra). Long oligocholates such as **4** prefer folding instead of aggregation in the presence of SDS because the rigid and awkwardly shaped steroid backbone prevents their tight/stable packing.^{23b} Assuming that the FRET in 1 mM SDS solutions comes from folding, the above data suggest that a significant population of unfolded conformer exists in **2** in 1 mM SDS.

Figure 5a plots F_{300}/F_{340} of **1–4** as a function of SDS concentration. With an increase in SDS, FRET becomes less efficient and drops most dramatically over 4–10 mM for **1–3**,

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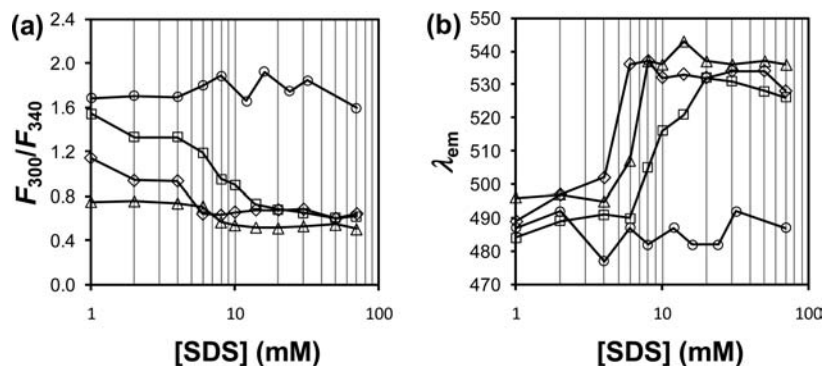


Figure 5. (a) F_{300}/F_{340} and (b) maximum emission wavelength (λ_{em}) of the dansyl in **1** (Δ), **2** (\square), **3** (\diamond), and **4** (\circ) as a function of SDS concentration. The data for **4** (\circ) were taken from ref 23b; [oligomer] = 2.0 μ M; λ_{ex} = 340 nm. The data points are connected to guide the eye.

as SDS begins to form micelles (cmc = 8 mM).²⁴ The behavior is very different for nonionic **4** (\circ), known to be folded below and above the critical micelle concentration (cmc).^{23a} It seems that micellization of SDS is very detrimental to the salt-bridged foldamer. The most likely cause for this effect is the sulfate headgroup of the surfactant, which can compete with carboxylate for the salt bridge. Below the cmc, although ionic surfactants such as SDS can assemble around large hydrophobic molecules²⁵ such as oligocholates, the number of surfactants around the foldamers must be limited. Upon micellization, a large number of surfactants assemble cooperatively to form a “community”. The main difference between the micelle and the individual surfactants is the much stronger solubilizing power of the former for hydrophobic guests and the higher local concentration of ionic headgroups. For nonionic **4**, micellization makes no difference because the molecule is overall hydrophobic and prefers to reside within the core of the micelle. The size of the micelle and the strong preference of the SDS micelle to maintain its spherical shape constrain the chain into the folded conformation.²³ For the salt-bridged **1–3**, two factors could disfavor the folded form. First, the high concentration of sulfate headgroups in the micelle can easily break the salt bridge. The solution studies above indicate that all three foldamers benefit to some extent from the salt bridge. Second, ionic groups such as guanidinium and carboxylate have a strong demand for solvation in water. Below the cmc, even though SDS molecules may assemble around the folded oligocholates, the assembly may be loose enough to allow water molecules to approach the ionic groups. Upon micellization, the oligocholate suddenly finds itself in a hydrophobic microenvironment. If solvation of the ionic groups is important to the overall system, the helix probably prefers to unfold and migrate to the surface of the micelle, where the ionic groups can be exposed.

The maximum emission wavelength (λ_{em}) of dansyl is sensitive to its microenvironment.¹⁸ Figure 5b plots the λ_{em} of **1–4** in different SDS solutions. In agreement with the folding–unfolding transition proposed above, all three salt-bridged foldamers emit at shorter wavelength below the cmc. The data indicate that the dansyl of these oligocholates is located in a relatively nonpolar microenvironment—this should correspond to the folded conformer surrounded by some SDS

molecules. Micellization causes a step change in the emission wavelength of dansyl, jumping from <500 to 530–540 nm. The data might be surprising given that micellization creates a hydrophobic microenvironment around the oligocholate, but they are quite reasonable if the oligocholate unfolds and migrates to the micellar surface. Consistent with the folding of **4**, its λ_{em} , although fluctuating to some extent, shows the dansyl being located in similar microenvironments below and above the cmc of SDS.

Because of the interference of the sulfate headgroup of SDS with the salt-bridge formation, we also studied the conformation of these compounds in Brij 35 solutions. The nonionic surfactant has the same dodecyl chain as SDS but an oligo(ethylene glycol) headgroup instead of sulfate. It is commonly used to solubilize membrane proteins,²⁶ making it particularly interesting to understand its effect on the oligocholates.

Figure 6a shows F_{300}/F_{340} of **1–4** in Brij 35. The oligocholates clearly behave differently in the presence of the nonionic surfactant. The F_{300}/F_{340} ratio for **1**, for example, is completely independent of the surfactant concentration, averaging 0.79 over 0.025–20 mM of Brij 35. The value is almost identical to that of the folded **1** in solution (0.78, Figure 3a), suggesting that the energy transfer comes from folding. Because the oligocholate is insoluble in water, the nonionic surfactant is basically the medium for the foldamer. If FRET stays unchanged over 8000-fold dilution (from 0.0025 to 20 mM of surfactant), there is very little likelihood that it could come from intermolecular aggregation. The cmc of Brij 35 is about 0.1 mM.²⁷ Hence, micellization of the nonionic surfactant does not unfold compound **1**.

The starting F_{300}/F_{340} for oligocholate **2** is 2.01 (Figure 6a, \square), exactly the same as that of the folded conformer in solution (Figure 3a, \square). Thus, the salt-bridged foldamer is fully folded in low Brij solutions. Its stability seems to be slightly lower than that of **1**, as the F_{300}/F_{340} ratio does show some decrease above the cmc of Brij 35 but still averages at about 1.6. This value is significantly higher than that for the unfolded (F_{300}/F_{340} = 0.48, Figure 3a) and even larger than the beginning value in SDS solutions (F_{300}/F_{340} = 1.54, Figure 5a). Apparently,

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(25) Hydrophobic polymers are known to be highly effective at inducing the aggregation of ionic surfactants below the cmc. See: Rosen, M. J. *Surfactants and Interfacial Phenomena*, 2nd ed.; Wiley: New York, 1989; p 181.

(26) (a) Yoshikawa, S.; Tera, T.; Takahashi, Y.; Tsukihara, T.; Caughey, W. S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 1354–1358. (b) Shinzawa-Itoh, K.; Ueda, H.; Yoshikawa, S.; Aoyama, H.; Yamashita, E.; Tsukihara, T. *J. Mol. Biol.* **1995**, *246*, 572–575. (c) Berger, B. W.; Garcia, R. Y.; Lenhoff, A. M.; Kaler, E. W.; Robinson, C. R. *Biophys. J.* **2005**, *89*, 452–464.

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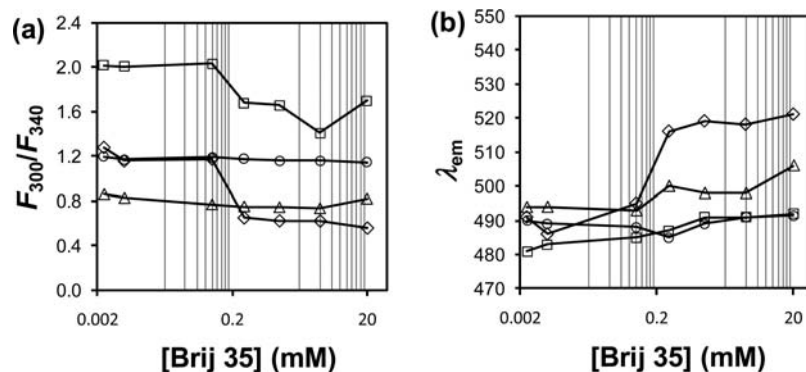


Figure 6. (a) F_{300}/F_{340} and (b) maximum emission wavelength (λ_{em}) of dansyl **1** (Δ), **2** (\square), **3** (\diamond), and **4** (\circ) as a function of Brij 35 concentration. [Oligomer] = 2.0 μ M; λ_{ex} = 340 nm. The data points are connected to guide the eye.

without the sulfate headgroup, the nonionic surfactant represents a much more folding-friendly environment to the salt-bridged oligocholates.

The charged oligocholate **3** is the only one being unfolded upon micellization. The F_{300}/F_{340} curve shows a distinct drop from 1.20 to about 0.6 around the cmc of Brij 35. When the three salt-bridged foldamers are compared, the effect of micellization on the conformational stability follows the order of **3** > **2** > **1**. The order can be rationalized by the increase in environmental hydrophobicity during micellization mentioned earlier. Compound **3** has a net positive charge and thus the strongest need for water solvation. Keeping it folded inside the micelle will be most difficult. Both **1** and **2** are overall neutral, but the former has higher inherent foldability according to the studies in homogeneous solutions (Table 1).

The F_{300}/F_{340} curve for **4** is completely flat (Figure 6a, \circ). The concentration-independent FRET is strong evidence for the folding of the oligocholate. The average F_{300}/F_{340} (1.17) is significantly lower than that (1.68) in the SDS micelles. It is not very clear why the nonionic oligocholate folds worse in the Brij micelles, but the result is still in line with the environmental hydrophobicity. Folding of **4** creates a hydrophilic cavity that prefers to be filled with water. It is clearly easier to put water molecules inside SDS instead of Brij micelles. Nonionic micelles are “drier” than ionic micelles in the interior.²⁸ Water is actually known to penetrate into SDS micelles appreciably.²⁹

Folding of Oligocholates in DLPC Bilayers and Translocation of Carboxyfluorescein across Lipid Membranes. Although surfactant micelles are frequently used to study membrane-associated proteins and peptides,¹¹ lipid bilayers differ significantly from surfactant micelles. They are liquid crystalline, anisotropic, and much more hydrophobic than most micelles. Because of the steroidal backbone, cholate derivatives are “naturally designed” to be compatible with lipids. Cholate derivatives, for example, are used in nature in fat digestion and lipid transport.³⁰ Regan and co-workers, in a series of seminal

work, described a cholate-based “molecular umbrella” for molecular transport across lipid bilayers.³¹ In this section, we report the conformational study of the oligocholates in membranes and our efforts in converting the oligocholates into useful molecular transporters.

Our initial assumption was that the salt-bridge would continue to be important to the conformation of oligocholates **1–3** in lipid membranes. Although the guanidinium–carboxylate interaction is weak in water, its association constant can be as high as 10^6 M^{-1} at the air–water or lipid–water interface.³² None of these oligocholates have any significant solubility in water. Thus, any salt-bridge interactions for the oligocholates must happen in or on the surface of lipid bilayers.

We employed the detergent dialysis method to incorporate the oligocholates into lipid bilayers.³³ This procedure is often employed to reconstitute membrane proteins into liposomes.³⁴ Briefly, we first solubilized the oligocholates in mixed micelles formed between Brij 35 and dilauroylphosphatidylcholine (DLPC). DLPC was chosen because it has the same C12 chains as SDS and Brij 35; we reasoned that the dimension of the hydrophobic environment should be important to the folding. As Brij 35 was removed from the mixed micelles by hydrophobic Bio-Beads, the remaining DLPC spontaneously forms liposomes with the oligocholates embedded in the membranes.

Table 2 summarizes the F_{300}/F_{340} ratio of these compounds in DLPC bilayers. To understand whether the observed FRET comes from folding or intermolecular aggregation, we varied the ratio between the oligocholate and DLPC from 1/50 to 1/1000. If folding is the dominant contribution to the observed

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(31) For other cholate-based molecular transporters, see: (a) Janout, V.; Di Giorgio, C.; Regen, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2671–2672. (b) Janout, V.; Staina, I. V.; Bandyopadhyay, P.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 9926–9927. (c) Janout, V.; Jing, B. W.; Staina, I. V.; Regen, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 4436–4437. (d) Janout, V.; Regen, S. L. *J. Am. Chem. Soc.* **2005**, *127*, 22–23. (e) Mehiri, M.; Chen, W.-H.; Janout, V.; Regen, S. L. *J. Am. Chem. Soc.* **2009**, *131*, 1338–1339.

(32) Ariga, K.; Kunitake, T. *Acc. Chem. Res.* **1998**, *31*, 371–378.

(33) We initially employed the membrane extrusion method to prepare oligocholate-containing liposomes. The F_{300}/F_{340} ratio in some oligocholates was higher at lower [oligocholate]/[lipid] ratio. This result was contrary to what intermolecular aggregation would produce and had been thought to support the folding of the oligocholates in the DLPC bilayers. Later, we found that, at high [oligocholate]/[lipid] ratio, the DLPC bilayers did not have the capacity to incorporate all oligocholate molecules into the bilayers, and the oligocholates were removed by the polycarbonate membrane used during extrusion. The detergent dialysis method had no such problems.

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Table 2. F_{300}/F_{340} Values for **1–4** in DLPC Bilayers^a

compound	F_{300}/F_{340}		
	[oligocholeate]/[lipid] = 1/50	[oligocholeate]/[lipid] = 1/200	[oligocholeate]/[lipid] = 1/1000
1	0.62	0.43	0.49
2	1.29	0.52	0.59
3	0.68	0.43	0.46
4	1.07	0.85	0.47

^a Determined from the excitation spectra of the liposomal solutions.

FRET, F_{300}/F_{340} should be independent of oligocholeate concentration in the lipid bilayer. If, on the other hand, aggregation is the dominant process, F_{300}/F_{340} should decrease upon lowering the [oligocholeate]/[lipid] ratio.

Unfortunately, all compounds seem to aggregate at high concentrations but dissociate and unfold at low concentrations in DLPC bilayers. FRET becomes less efficient in all oligocholeates at lower [oligocholeate]/[lipid] ratio. The spectra for [oligocholeate]/[lipid] = 1/1000 were noisier than others due to the extremely low concentration of the fluorophores; thus, the small increase from 1/200 to 1/1000 in some F_{300}/F_{340} could very well be an artifact. The 1/1000 liposomes represent the least likely environment for aggregation (and thus most likely for folding), yet F_{300}/F_{340} ranged from 0.46–0.59, clearly corresponding to the unfolded structures.

It is not difficult to understand why the oligocholeates do not want to fold in DLPC bilayers. From the conformational studies in micelles, we know that both salt-bridge-breaking groups and high environmental hydrophobicity are detrimental to the folding. A phospholipid bilayer certainly represents the worst combination. The surface of the membrane is densely packed with phosphate groups, which are very effective at hydrogen-bonding with the guanidinium group.²¹ The interior of a lipid bilayer is much more hydrophobic than that of a micelle. Solvation of the introverted polar groups (hydroxyl and amide on the cholate and the amino acid side chains) of the folded helix will be significantly more difficult in a lipid bilayer as a result.

Undeterred by the apparent failure, we decided to investigate the potential of these oligocholeates as molecular transporters. Even though the FRET studies indicate that the molecules are unfolded in the lipid bilayers, they do not mean that the oligocholeates cannot fold in a transient fashion. The folded helix (Scheme 1) has a hydrophobic exterior and a nanometer-sized hydrophilic cavity, perfect for shielding a hydrophilic guest and escorting it across a hydrophobic barrier. This is a highly attractive application, as selectively transporting hydrophilic molecules across cell membranes is extremely important to applications such as drug delivery.

The membrane transport was based on the well-established carboxyfluorescein (CF) leakage assay.³⁵ CF is a water-soluble dye that displays self-quenching at high concentrations. Large unilamellar vesicles (LUVs) consisting of POPC/POPG lipids were prepared by standard methods in the presence of 50 mM CF. DPLC could not be used in this experiment because the liposomes were too unstable to trap CF. The extravesicular CF was removed by gel filtration. Leakage was monitored by the increase of CF fluorescence upon injection of the oligocholeates in DMSO. The efflux was followed for 60 min, at which the LUVs were lysed by the addition of Triton X-100.

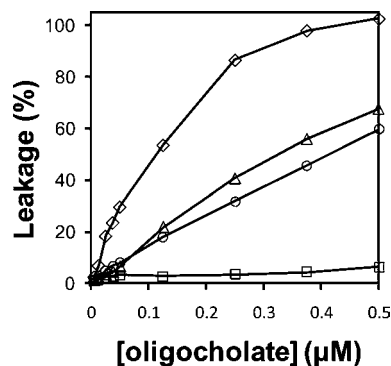


Figure 7. Percent leakage of CF from LUVs for **1** (Δ), **2** (\square), **3** (\diamond), and **4** (\circ) as a function of oligocholeate concentration.

To our delight, the oligocholeates are quite effective transporters of CF, and the leakage is strongly affected by their functionality. The compound with the extra arginine (**3**) is the best transporter among the four, causing nearly 90% of the entrapped CF to leak out at 0.25 μM concentration (Figure 7). The strong transporting ability probably derives from its ability to bind CF, which contains two carboxylic acid groups. Binding, however, is not absolutely necessary, as the nonionic **4** shows about half of the activity. Presumably, the oligocholeate can fold into the helix and the hydrophilic cavity is large enough to accommodate CF. The oligocholeate does not have to be folded permanently for the transport; as long as the molecule can fold and migrate across the membrane, it should be able to transport hydrophilic guests. According to the CPK model, a fully folded cholate hexamer is about 1.5 nm in length. The hydrophobic thickness of the POPC bilayer is about 2.6 nm.³⁶ The nearly linear relationship between the efflux and the oligocholeate concentration suggests that **4** probably acts as a molecular carrier. It should be mentioned that foldability of the oligocholeates was important to the transport, as methyl cholate and shorter oligomers (e.g., dimer and trimer) displayed no activity at all (data not shown). Other mechanisms such as liposome fusion were excluded by lipid mixing assays (Figure 4S, Supporting Information).

The most striking difference in Figure 7 is between **1** and **2**. Although compound **1** displays transport comparable to that of the non-salt-bridged **4**, **2** is completely incompetent. The conformational studies in homogeneous solution indicate that **2** has a lower inherent foldability than **1**. The micellar studies also suggest that **2** is more susceptible to salt-bridge-breaking groups and environmental hydrophobicity. The difference must be large enough to devoid **2** of its ability to fold even transiently within the membrane.

Conclusions

Although it is convenient to study the conformation of proteins and peptides in homogeneous solution and/or micelles, great care must be taken to directly extrapolate the results obtained in one environment to another. Such a treatment assumes that the inherent foldability of foldamers stays the same across different environments, which clearly is not the case for the oligocholeates. Environmental effects can easily overwhelm the inherent foldability of the molecules. As the surrounding of the molecule is changed and different noncovalent forces

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are involved in the conformational control, the best folder (i.e., **3**) could become the worst, and the worst (i.e., **4**) could become the best.

Folding of the oligocholates in homogeneous solutions is dominated by the preferential solvation. This mechanism is an interesting variation of solvophobic interactions. In conventional solvophobic interactions, poor solvation of a molecular surface (e.g., hydrocarbon by water) causes the molecule to aggregate to minimize solvent exposure. Although the same is true for the hydrophilic faces of the cholates groups in **1–4**, the hydrophilic faces cannot aggregate directly due to the rigidity of the backbone. Instead, the phase-separated methanol acts as a bridge to hydrogen-bond with the hydroxyl and amide groups. It is interesting that even a non-participating arginine can strongly influence the conformation of the molecules.

As the molecules move into micelles, preferential solvation by different solvents is no longer available, but the need for the amphiphilic molecules to interact with appropriate environments stays the same. The solvation of ionic groups by water is a strong factor controlling the molecular conformation, and the salt bridge continues to be important. The folded helix demands its interior to be solvated by a pool of water molecules. Folding, as a result, becomes more difficult as the oligocholates move from ionic SDS micelles to nonionic Brij 35 micelles. Although the zwitterionic **1** and **2** can still fold in the latter, the cationic **3** lost its ability to fold in the more hydrophobic microenvironment.

The phosphate groups and hydrophobicity of the lipid bilayer make it the most challenging environment for the oligocholates.

The FRET studies indicate that the molecules aggregate at higher concentrations (e.g., at [oligocholate]/[lipid] = 1/50) and the aggregates dissociate at low concentrations (e.g., at [oligocholate]/[lipid] = 1/200–1/1000). It is not exactly clear what the molecules do on or in the membrane. The FRET shows that the unfolded conformers dominate. The molecules probably are embedded to a certain extent in the membrane, considering their strong hydrophobicity. The molecules, however, can readily fold and migrate to the other side of the membrane, evidenced by their ability to transport large water-soluble guests across lipid bilayers. The transport efficiency depends on both the foldability and the binding with the guest. The modular synthesis of the oligocholates, the predictability of their conformational behavior, and the ease of their functionalization by natural α -amino acids potentially will make them very useful, selective molecular transporters.

Acknowledgment. This paper is dedicated to Prof. Joseph B. Lambert for his 70th birthday. We thank the U.S. Department of Energy, Office of Basic Energy Sciences (grant DE-SC0002142), for supporting the research.

Supporting Information Available: Experimental Section, including the general experimental details, synthesis and characterization of the compounds, data analysis, and fluorescence and lipid-mixing data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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