α-Helix Formation in Melittin and β-Lactoglobulin A Induced by Fluorinated Dialcohols

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Extensive study of the effect of fluorinated alcohols on protein conformations, notably the induction of α-helix formation, is important because of its wide range of applications. Circular dichroism (CD) was used to show that the enhancement of helix induction in β-lactoglobulin A and melittin by the fluorinated diols 2,2,3,3-tetrafluoro-1,4-butanediol (TFBD), 2,2,3,3,4,4-hexafluoro-1,6-pentanediol (HFPD), and 2,2,3,3,4,4,5,5-octafluoro-1,6-hexanediol (OFHD) increases in the order TFBD < HFPD < OFHD. For fluorinated diols and monoalcohols the effectiveness of helix induction was found to increase exponentially with increasing number of fluorine atoms per alcohol molecule, and OFHD was found to be more effective than any previously reported fluorinated alcohol. Formation of standard micelles was ruled out as the cause of the enhanced helix induction by the fluorinated diols. The negligible red-edge excitation shift in the fluorescence of melittin indicated that the fluorinated diol/water solvent shell surrounding the tryptophan chromophore is less immobilized than are molecules in a lamellar vesicle.

Introduction

It is known that alcohols, notably 2,2,2-trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), induce the conformational transition of globular proteins into predominantly secondary structure (primarily α-helices).[1−3] These effects have been used in several ways. The secondary structures stabilized in alcohols have been proposed to serve as the initiation site in studies of protein folding.[4,5] TFE and HFIP have been used to dissolve aggregates that occasionally form during peptide synthesis. Also, HFIP has been used to disaggregate Alzheimer's amyloid peptides and prion disease peptides.[6−10] The ability of TFE to penetrate into the hydrocarbon phase of micelles is believed to promote incorporation of peptides into a membrane.[11] Cosolvents have been used to dissect and reassemble amyloid fibrils.[12] The influence of cosolvents on the folding kinetics of proteins has been studied.[13,14] Glycerol, a triol, is frequently used to stabilize proteins, and 2-methyl-2,4-pentanediol has been used to crystallize proteins.[15] A comprehensive description of the effects of TFE and similar cosolvents on polypeptide chains has been given.[1]

Several factors responsible for the helicogenicity enhancement have been identified. Alcohols decrease the local dielectric constant[16] and weaken hydrophobic interactions stabilizing the native structure and increase helical propensities, which disrupts the native tertiary structure.[17] It has been shown that for some repeating-sequence peptides, containing mainly alanine residues, the presence of TFE strengthens hydrogen bonds within the helical state to an extent sufficient to account for the increase in helix propensity in the presence of TFE.[18] An indirect mechanism may be involved in which peptide hydrogen bonds to the solvent are weakened in conjunction with lowering of the solvent dielectric constant.[1,19−21] Alternatively, helicogenic enhancement may occur by a direct mechanism involving preferential solvation of the polypeptide and direct binding of TFE to the polypeptide chain in the so-called helical state.[22−25] One hypothesis is that the helix−coil equilibrium is shifted toward the helical form due to the preferential binding of TFE to the helical form.[3] To emphasize the importance of hydrophobic interactions, it has been proposed that TFE can take advantage of an extensive hydrophobic α-helix surface by preferentially binding to the amphiphilic helical structure.[26]

An important step in trying to understand the helicogenicity enhancement caused by alcohols was the work of Hirota et al., who showed that contributions to the efficiency of helix induction by different chemical groups (i.e., CH, OH, and F) are additive.[27] Important exceptions to the group additivity concept appear to be 2-fluoroethanol, TFE, and HFIP, which enhance helix formation at much lower alcohol concentrations than are required for alkanols.[26,27] One purpose of the work described herein is to extend the results reported for 2-fluoroethanol, TFE, and HFIP to fluorinated diols and to show explicitly for six fluorinated diols and monoalcohols that *m* (a measure of relative helix enhancement) increases exponentially with the increasing number of fluorine atoms.

The exceptional helicogenicity enhancement by HFIP and TFE suggests the role of additional factors for these two alcohols and continues to be of interest.[28] One suggested factor[2,27] is the tendency of TFE and especially HFIP to form clusters in aqueous solution as a way to reduce contact between the hydrophobic fluorocarbon part of the molecules with polar water molecules.[29,30] It has been proposed that the matrix provided by such clusters assists local secondary structure folding by promoting interactions between hydrophobic amino acid side chains.[31] In fact, plots of the transition curve (for conversion of the polypeptide into α-helix) vs X-ray scattering intensity for solutions containing melittin and either TFE or HFIP suggested that clustering is a significant factor responsible for the marked effects of TFE and HFIP.[2] However, this result is contradicted by the observation that helix induction by TFE and HFIP in phosphoglycerate kinase and β-lactoglobulin A begins to occur at concentrations well below the critical concentration at which cluster formation sets in.[32] This observation shows that cluster formation only accompanies but is not primarily

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responsible for helix induction, and the results suggest that the extraordinary potential of HFIP arises due to extensive preferential binding.[32] Although this apparent difference between whether or not cluster formation is essential for helix induction by TFE and HFIP may simply be due to the difference between the proteins, it is hard to rationalize a change in the mechanism for TFE and HFIP with the nature of the protein.

If formation of clusters is important for helix induction, it is important to characterize them. Small-angle scattering results have shown that in alcohol/water mixtures HFIP has a tendency to form assemblies that are micelle-like in nature.[33] In aqueous solution alcohols form what are referred to as micelle-like assemblies with the hydrophobic groups inside.[34,35] One purpose of the studies described herein is to show that helix induction by the fluorinated diols 2,2,3,3-tetrafluoro-1,4-butanediol (TFBD), 2,2,3,3,4,4-hexafluoro-1,5-pentantdiol (HFPD), and 2,2,3,3,4,4,5,5-octafluoro-1,6-hexanediol (OFHD) cannot involve formation of micelle-like clusters since these alcohols cannot form micelles due to the symmetric placement of hydroxyls on each end of the molecule.

The other purposes of the experiments described herein are to (1) increase the number of fluorinated alcohols about which data on helicogenesis enhancement is available, (2) do the first systematic study of the enhancement of helix formation induced by fluorinated diols, (3) find an alcohol that induces helix formation at lower concentrations than are required for TFE and HFIP, which could be of significance in situations that require minimal concentrations of helix inducer, (4) determine whether fluorinated diols show the extraordinary helix induction of fluorinated monoalcohols and demonstrate more clearly and extend the direct evidence that the induction effects of fluorinated alcohols are more than linearly additive, which requires a comparison of three or more alcohols, and (5) compare the effectiveness of fluorine atoms that are bonded to terminal carbons and interior carbons of alcohol molecules.

The protein β-lactoglobulin A and polypeptide melittin were studied because they represent two quite different models for the conformational transition to helical form. β-Lactoglobulin A has a molar mass of 18.4 kD, contains two disulfide bonds and one cysteine residue, and is a predominantly β-sheet protein consisting of nine antiparallel β-strands and one α-helix that is in the monomeric, compactly folded native state in 10 mM HCl.[36] Melittin is a major component of honeybee venom and consists of 26 amino acids, including a single tryptophan at residue 19. At pH 2 melittin exists as an unfolded monomer. It is believed that in a physiological system melittin folds into a monomeric α-helix upon contact with the membrane surface, similar to the α-helix induction that occurs upon interaction with micelles.[37] For melittin the conformational transition to α-helix involves the formation of an organized secondary structure, which is stabilized by its internal hydrogen bonds, without the need for the disruption of internal interactions within the initial state. In contrast, in β-lactoglobulin A internal interactions in the organized structure of the initial state must first be overcome during the conformational transition.

Experimental Section

Chemicals. Melittin and β-lactoglobulin A were obtained from Sigma-Aldrich and were used without further purification. The supplier stated the minimum purities, based on HPLC measurements, to be 93% for melittin and 90% for β-lactoglobulin A. All fluorinated diols and 2-propanol were of highest purity, obtained from Sigma-Aldrich, and were used without further purification.

Instrumentation and Sample Preparation. Circular dichroism (CD) spectra were recorded at 25 °C with a Jasco model J-715 spectropolarimeter. A cylindrical cell with a 1-mm path length was used, and the protein concentration was 0.1 mg/mL. Circular dichroism signals were recorded as molar ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters.

The conformational transition of melittin and β-lactoglobulin A was followed by measuring the far-UV CD spectra as a function of concentration of alcohol contained in 10 mM HCl solution. Typically, 30 μL of protein solution in 10 mM HCl was mixed with 470 μL of 10 mM HCl solution containing different alcohol concentrations to achieve a final polypeptide concentration of 0.1 mg/mL. The highest concentration of each alcohol used to make CD measurements was limited by its solubility. Since the alcohol-induced conformational transition occurs rapidly, a circular dichroism spectrum was obtained within a few minutes after each sample was prepared.

Fluorescence spectra were recorded at 25 °C with a Jobin Yvon/Horiba fluoromax-3 spectrofluorometer, using 1-cm path length quartz cuvettes. Excitation and emission slits were set to a band-pass of 2.5 nm for all measurements, and the integration time was set in the range 0.5−2.0 s, depending on the strength of the fluorescence. The fluorescence spectrum for each sample was corrected for the solvent Raman peak and artifactual solvent emission by subtracting the emission spectrum acquired for the corresponding solution not containing melittin. Red-edge excitation was limited to wavelengths less than or equal to 309 nm due to the difficulty at higher wavelengths associated with obtaining proper subtraction of fluorescence from an impurity contained in the fluorinated diols. Typically, 50 μL of protein solution in 10 mM HCl was mixed with 1.95 mL of 10 mM HCl solution containing different alcohol concentrations to achieve a final polypeptide concentration of 0.25 mg/mL.

Results and Discussion

Analyses of Changes in Circular Dichroism Signal with Alcohol Concentration. The conformational transition is assumed to involve only two states for both β-lactoglobulin and melittin. Justification for the use of this assumption is given in a later section. During the conformational transition melittin passes between unfolded and helical states, U $\rightleftarrows$ H, and β-lactoglobulin goes from the native state to the helical state, N $\rightleftarrows$ H. The free energy change for the transition is calculated from $\Delta G_H = -RT$ ln[helical form]/[initial form] and is found experimentally (data not shown) to vary linearly with alcohol concentration (i.e., $\Delta G_H = \Delta G_H(H_2O) - m$[alcohol], where $\Delta G_H(H_2O)$ is the free energy change extrapolated to zero alcohol). m is a measure of how strongly ΔG_H depends on the alcohol concentration and relates to the effectiveness of helix induction by an alcohol and the cooperativity of the conformational transition. Although the linearity of ΔG_H vs alcohol concentration has not been established as generally applicable, such analysis has been used by others.[3,26]

A standard method is used to analyze the CD spectral data.[38] The concentration ratio [helical form]/[initial form] is obtained from the molar ellipticity at 222 nm. The CD signal is assumed to vary linearly at low and high alcohol concentrations in accordance with $a_N + b_N$[A] and $a_H + b_H$[A], respectively. It follows that $\Delta G_H(H_2O) = mC_m$, where C_m designates the alcohol concentration at the midpoint of the conformational transition. By combining this expression and the two expressions in the

TABLE 1: C_m, m, and $\Delta G(H_2O)$ Values for the Alcohol-Induced Conformational Transitions of Melittin and β-Lactoglobulin A at pH 2.0 and 25 °C[a]

	melittin			β-lactoglobulin A		
alcohol	C_m (M)	m (kJ/mol/M)	$\Delta G(H_2O)$ (kJ/mol)	C_m (M)	m (kJ/mol/M)	$\Delta G(H_2O)$ (kJ/mol)
TFBD	1.41	6.9	9.7	2.42	7.7	19
HFPD	0.64	15	9.6	0.85	35	30
OFHD	0.22	40	8.8	0.31	88	27

[a] The values of C_m and m were determined by fitting of experimental data to eq 1 with the aid of a nonlinear least-squares routine. $\Delta G(H_2O) = mC_m$. The standard deviations were less than 10% for all C_m and m values, except for the β-lactoglobulin A value of m, which was 19%, apparently due to the poorly formed maximum of the curve in Figure 2.

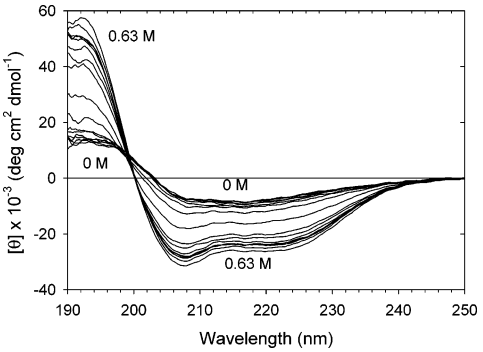

Figure 1. Far-UV CD spectra of β-lactoglobulin A in solutions containing different concentrations of OFHD at pH 2 and 25 °C. The lowest and highest concentrations are indicated and are 0 and 0.63 M, respectively.

previous paragraph, eq 1 is derivable. The experimental values of $[\theta]_{222}$ were fitted to eq 1 by a nonlinear least-squares program included with Mathcad software. Since for all three fluorinated diols the experimental intercepts of $[\theta]_{222}$ plotted vs fluorinated diol concentration were nearly identical (see Figures 2 and 4), the value of a_N was held constant and equal to 4.3 mdeg cm^2 dmol^{-1} and 6.2 mdeg cm^2 dmol^{-1} for melittin and β lactoglobulin A, respectively. The fitting procedure gave values and standard deviations for a_H, b_H, b_N, C_m, and m, from which $\Delta G_H(H_2O)$ was determined. Values of C_m, m, and $\Delta G_H(H_2O)$ are reported in Table 1.

$$[\theta]_{222} = \frac{(a_N + b_N[A]) + (a_H + b_H[A])\exp\{(m[A] - mC_m)/RT\}}{1 + \exp\{(m[A] - mC_m)/RT\}} \quad (1)$$

Fluorinated Diol-Induced Conformational Transition of β-Lactoglobulin A and Melittin. To ensure consistency between the results reported herein and in the literature, a plot of $[\theta]_{222}$ vs 2-propanol concentration (data not shown) was constructed from which the C_m value was found to be 2.4 M for melittin, in agreement with ref 26.

Figure 1 shows the far-UV CD spectra of β-lactoglobulin A in solutions containing different concentrations of OFHD. Similar spectra were obtained for β-lactoglobulin A in solutions containing different concentrations of TFBD and HFPD. The spectra in Figure 1 are very similar to those reported for helix induction by TFE and HFIP and contain the same isodichroic region of 199−200 nm.[26] The CD spectrum corresponding to zero alcohol shows a single minimum, indicative of a predominantly β-sheet structure. Addition of alcohol dramatically changes the spectrum to that corresponding to an α-helix with minima at 208 and 222 nm.

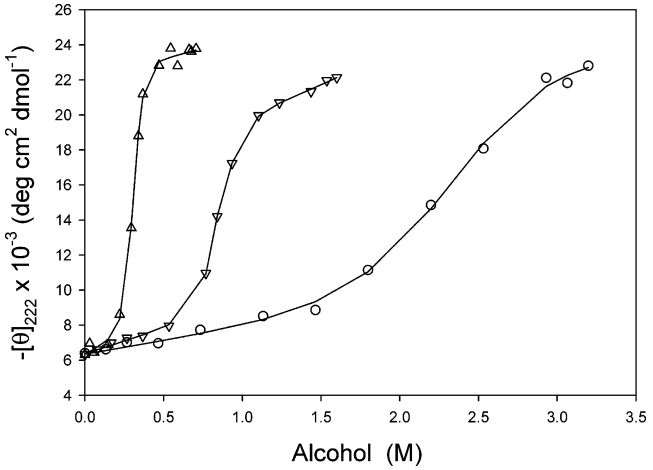

Figure 2. Alcohol-induced conformational transition of β-lactoglobulin A measured by the molar residue ellipticity at 222 nm as a function of alcohol concentration: △, OFHD; ▽, HFPD; ○, TFBD. The fits of eq 1 to the data are given by the solid curves.

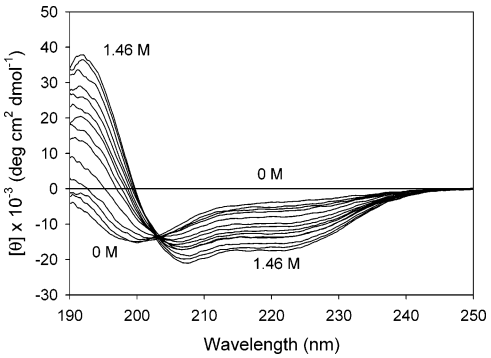

Figure 3. Far-UV CD spectra of melittin in solutions containing different concentrations of HFPD at pH 2 and 25 °C. The lowest and highest concentrations are indicated and are 0 and 1.46 M, respectively.

For β-lactoglobulin A the dependence of $[\theta]$ at 222 nm on alcohol concentration for all three fluorinated diols is shown in Figure 2 in which the solid lines have been produced by fitting the data to eq 1. The similar maximum ellipticities indicate that the maximum helical content is about the same for all three fluorinated diols. The helical content is estimated by the method of Chen et al.[39] to be a maximum of 80%, which is similar to the value reported for helix induction by TFE and HFIP.[26] Comparison of the C_m values for β-lactoglobulin A, given in Table 1, shows that they decrease faster than linearly with respect to the number of fluorines in the alcohol molecule.

Figure 3 shows the far-UV CD spectra of melittin in solutions containing different concentrations of HFPD. Similar spectra were obtained for melittin in solutions containing different concentrations of TFBD and OFHD. The spectra in Figure 3 are very similar to those reported for helix induction by TFE and HFIP and contain the same single sharp isodichroic point at 203 nm.[26] The CD spectrum corresponding to zero alcohol is similar to that for a so-called random coil. Addition of alcohol changes dramatically the spectrum to that corresponding to an α-helix with minima at 208 and 222 nm.

For melittin the dependence of $[\theta]$ at 222 nm on alcohol concentration for all three alcohols is shown in Figure 4 in which the solid lines have been produced by fitting the data to eq 1. The similar maximum ellipticities indicate that the maximum helical content is approximately the same for all three fluorinated diols. The helical content is estimated by the method of Chen

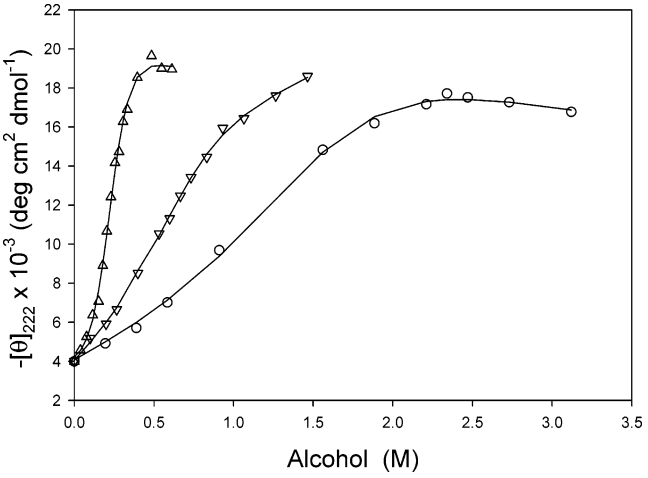

Figure 4. Alcohol-induced conformational transition of melittin measured by the molar residue ellipticity at 222 nm as a function of alcohol concentration: △, OFHD; ▽, HFPD; ○, TFBD. The fits of eq 1 to the data are given by the solid curves.

et al.[39] to be a maximum of nearly 70%, which is in agreement with the 70% value reported for helix induction by TFE and HFIP.[26]

Comparison of the Induction of the Conformational Transition in *β*-Lactoglobulin A and Melittin by Fluorinated Monoalcohols and Diols. The values of $[\theta]$ at 222 nm begin to increase substantially at the lowest alcohol concentrations in Figure 4 but increase more gradually at low alcohol concentrations before the onset of large changes in Figure 2. This is reasonable in view of the greater structural change that must occur in *β*-lactoglobulin, namely the disruption of intramolecular interactions, as it goes from the compact native folded state through an intermediate to the helical state. In contrast, for melittin no organized structure must be undone as it changes structurally from an unfolded form into the more compact helical structure. Similar behavior for plots of $[\theta]_{222}$ vs alcohol concentration was observed for helix induction by TFE and HFIP in *β*-lactoglobulin A and melittin.[26]

Figures 2 and 4 and the midpoint concentrations (C_m) of the transitions given in Table 1 show that the order of effectiveness of the fluorinated diols is TFBD < HFPD < OFHD for both *β*-lactoglobulin A and melittin. A similar increase in effectiveness with increasing number of fluorine atoms per alcohol molecule was reported for the fluorinated monoalcohols TFE and HFIP.[26] This same order of effectiveness occurs for both melittin and *β*-lactoglobulin A despite the necessity for disruption of intramolecular interactions during the initial stage of the conformational transition in *β*-lactoglobulin A. Thus, as has been pointed out for fluorinated monoalcohols,[26] this suggests that the denaturant potential of a fluorinated diol is correlated with the common step in the mechanism for melittin and *β*-lactoglobulin A, which is the stabilization of the helical conformation relative to the unfolded polypeptide. Moreover, since the step involving destabilization of the native state is necessary only in the mechanism for *β*-lactoglobulin A, the same order of alcohol effectiveness for melittin and *β*-lactoglobulin A indicates that the process of helix formation from the unfolded state is the same in both polypeptides.

Thermodynamic Aspects of the Two-State Model. For *β*-lactoglobulin A the assumed two-state model is not rigorous, and the existence of an intermediate has been reported.[40,41] For melittin it is also possible that the conformational transition is not simply two-state but may involve intermediate helical structures with frayed ends, such as those described for short helical peptides.[18,42−45] However, for melittin a very good correlation has been reported between results obtained through data analyses employing either a simple two-state model or the more realistic Lifson−Roig theory,[27,46] and it is expected that any attempt to apply Lifson−Roig theory herein would yield no substantive changes in the relative *m* values for the fluorinated diols in Table 1. Although the thermodynamics analysis is not rigorous, a two-state model was used by Hirota et al. and its use herein is only as extensive as the use made by Hirota et al.[27]

It is expected that the value of ΔG_H for the conformational transition to the α-helical state should have the same value when extrapolated to zero alcohol concentration. In agreement with this expectation, the values of $\Delta G_H(H_2O)$ in Table 1 for all three alcohols are approximately the same for both melittin and *β*-lactoglobulin A. The averages of the $\Delta G_H(H_2O)$ values in Table 1 are 9.4 and 25 kJ/mol for melittin and *β*-lactoglobulin A, respectively, and agree with the reported values of 9.5 and 23.3 kJ/mol.[26,47]

The values of *m* in Table 1 are larger for *β*-lactoglobulin A than for melittin, consistent with the greater cooperativity of the conformational transition in *β*-lactoglobulin A. Also, the fact that the C_m values are lower for melittin than for *β*-lactoglobulin A is consistent with the results reported.[26]

Use of the additivity rules established by Hirota et al. for melittin gives additional important insights and allows a comparison of results for fluorinated monoalcohols and fluorinated diols.[27] They found that hydroxyl groups diminish the effectiveness of helicogenesis induction by alcohols.[27] Therefore, to put the data reported by Hirota et al. for fluorinated monoalcohols and those reported herein on the same scale, the *m* values in Table 1 must be corrected higher by an amount equal to the reduction in *m* produced by the presence of a second hydroxyl group. The correction can be estimated with the aid of eq 1 in ref 27, which expresses *m* as the sum of linear terms that represent empirically determined contributions from CH, OH, and halogen groups. The contribution due to OH group(s) is expressed as the product *b* times ASA(OH), where *b* is determined empirically, and ASA(OH) equals the accessible surface area that the OH group(s) present to a water molecule as it "rolls" over the surface of the OH group(s). This product is different for all alcohols, but from Tables 2 and 3 in ref 27 it is seen to be in the range 1.19−1.31 kJ/mol-M for the primary alcohols *n*-propanol, ethanol, and methanol and in the range 2.07−2.37 kJ/mol/M for the primary diols 1,2-ethanediol, 1,3-propanediol, and 1,4-butanediol. The value of the difference between the averages of these two ranges (0.98 kJ/mol/M) then represents the amount by which the *m* value for an alcohol is reduced upon the addition of a second alcohol group. Figure 5 is a plot of the experimental *m* values reported for 2-fluoroethanol, TFE, and HFIP and the *m* values from Table 1, corrected by the addition of 0.98 kJ/mol/M, vs the number of fluorine atoms in each fluorinated alcohol (diol) molecule.[27]

Two important points are significant regarding Figure 5. First, the figure demonstrates directly for six alcohols that the effectiveness of fluorinated alcohols in inducing helix formation does not increase linearly but exponentially with increasing number of fluorine atoms. Second, the relatively smooth increase in the *m* value shows that the fluorinated monoalcohols and diols can be scaled together, which shows that the helix-induction effectiveness of fluorine atoms is about the same whether the fluorines are bonded to terminal carbons or interior carbons of an alcohol.

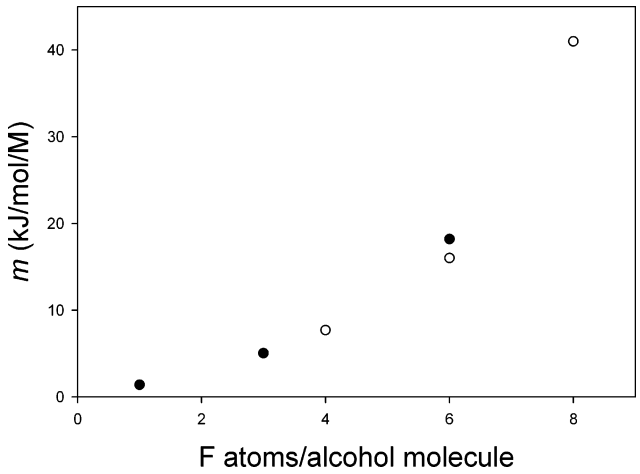

Figure 5. Dependence of the *m* value for the conformational transition in melittin on the number of fluorine atoms per alcohol molecule. The solid circles correspond to 2-fluoroethanol, TFE, and HFIP.

Comment on the Role of Micelle and Cluster Formation in Helix Induction. Although little is known about the internal nature of the clusters that have been reported to form in fluorinated alcohols, two categories are often discussed: micelle-like and clathrate-like clusters. Since the interior of both micelles and clathrate-like structures contains fewer water molecules than are present in a comparable volume of the bulk solvent, the interior has a lowered polarity. If the interior of such a clathrate-like structure or micellar structure envelops a portion of a polypeptide backbone, the favorable interactions between polypeptide and solvent are reduced in number and are replaced by intrapeptide hydrogen bonds between amide hydrogens and carbonyl oxygens, which are present in an α-helix. Consequently, the helical structure becomes statistically more favorable.

Amphipathic fluorinated monoalcohols can form typical micelles with polar surfaces (vide supra). However, the symmetric fluorinated diols reported herein are expected to have very small net dipole moments and cannot form micelles because the ends of the molecules are identical. So the significant conclusion can be drawn that if clusters are important to the process of helix enhancement by fluorinated diols, they cannot be typical micelles and should not be referred to as micelle-like in nature. It might be tempting to extrapolate this conclusion to the mechanism for helix induction by fluorinated monoalcohols since a common mechanism for helix induction in melittin by fluorinated monoalcohols[26] and the fluorinated diols reported herein is suggested by the similar trend in helicogenicity enhancement with increasing number of fluorines contained in the alcohol molecule, the similar extent of helix formation, the similar CD spectra with identical isodichroic points associated with the conformational process, and the apparent smooth scaling of *m* values in Figure 5. However, since these similarities are based only on the CD spectroscopy results, which relate only to the mechanism with regard to the protein and not the solvent, such extrapolation is invalid.

Hence, the mechanism with regard to the solvent may be different for fluorinated monoalcohols and fluorinated diols and may be related to the solubility differences between these two classes of alcohols. Unlike the fluorinated diols used in the work described herein, TFE and HFIP are miscible with water in all proportions and may have higher solubilities because they can form micelle-like clusters with polar surfaces. The nature of clusters that might form in fluorinated diols is not known, but at least two possible types are readily suggested. These molecules can minimize contact between water and their hydrophobic portions by forming extended arrays of molecules in which the fluorocarbon interior of each molecule must be juxtaposed on both sides with the fluorocarbon interior of other fluorinated diol molecules. Another possibility is that the hydroxyl groups on opposite ends of the molecules can form intermolecular hydrogen bonds to hydroxyls on other alcohol molecules. But whatever possible differences may exist in the mechanism due to the different solubilities of the alcohols, the point has nevertheless been established that for fluorinated diols the helix induction need not and cannot involve micelle-like clusters.

Because the ends of the fluorinated diol molecules are identical, under the appropriate experimental circumstances they could conceivably form monolayer vesicles, in which the interior hydroxyls gain thermodynamic stabilization by interacting with the aqueous pool trapped in the interior of the vesicle. However, since vesicle formation generally requires vigorous stirring, it is quite unlikely that the fluorinated diols could have formed vesicles in the experiments reported herein.

Qualitative insight regarding the degree of organization and mobility of the solvent environment surrounding the tryptophan chromophore of melittin can be obtained from a consideration of the red-edge excitation shift (REES) of the Trp fluorescence. The wavelength of maximum fluorescence emission for a polar fluorophore in a viscous solution or condensed phases with motionally restricted environments has been observed to shift to the red as the excitation wavelength is shifted toward the red edge of the absorption band.[48−55] This REES phenomenon occurs when the dipolar relaxation time for the solvent shell surrounding the fluorophore becomes comparable to or longer than the fluorescence lifetime. As an example, it is known that when melittin is bound to bilayer membranes, it is localized in the motionally restricted interfacial region of the membranes.[56−58] As a result, a REES of 5 nm, corresponding to the excitation wavelength range 280−310 nm, was observed for melittin bound to unilamellar vesicles of dioeoyl-*sn*-glycero-3-phosphocholine (DOPC) in aqueous solution.[57] An even larger REES of 4−8 nm, corresponding to the excitation wavelength range of 280−307 nm, was observed for melittin incorporated in reverse micelles of sodium bis(2-ethylhexyl) sulfosuccinate (AOT) that are formed in hexane solvent.[48] However, the dissimilarity of hexane and aqueous solvents makes difficult the comparison of the results reported in ref 48 and herein.

If the fluorinated diols form a sufficiently organized molecular assembly that envelops melittin in a way that localizes the tryptophan in a region where its reorientational motion is sufficiently restricted, then a REES should be observed. To test this possibility, the wavelength of the fluorescence maximum was measured as a function of the excitation wavelength for melittin contained in aqueous solutions (pH 2.0) of the fluorinated diols. The constancy of the fluorescence spectral profile for solutions with different concentrations of fluorinated diols showed that the tryptophan of melittin was the predominant fluorophore. For solutions not containing fluorinated diols, when the excitation wavelength was 280 nm, the wavelength for maximum fluorescence intensity, $I_{\max,\lambda}$, was observed to occur at 356 nm and not 353 nm as reported.[57] This difference is apparently due to the different solvent conditions—our solutions were unbuffered at pH 2.0, and the solutions used in ref 48 were buffered at pH 7 and contained 10 mM sodium phosphate/150 mM NaCl and 1 mM EDTA.

The results in Figure 6 show that no significant net REES is produced by 0.62 M OFHD. It is seen that over the excitation

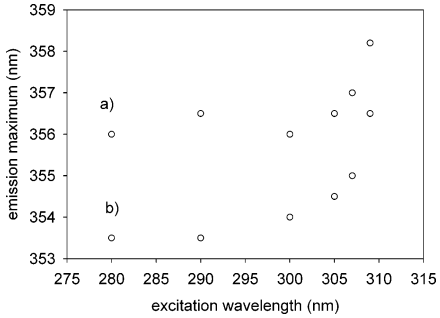

Figure 6. Effect of changing the excitation wavelength on the fluorescence maximum of melittin in (a) unbuffered pH 2.0 aqueous solution and (b) unbuffered pH 2.0 aqueous solution containing 0.62 M OFHD.

wavelength range 280−307 nm, melittin displays approximately the same REES of ~1.5 nm in aqueous solution as in 0.62 M OFHD. Only at 309 nm excitation does a small difference in REES become apparent, i.e., <3 nm vs >2 nm for 0.62 M OFHD and aqueous solution, respectively. If the magnitude of the REES is taken as the difference between the two plots in Figure 6, then the maximum net REES achieved is ≤1 nm. At lower OFHD concentrations the REES was even smaller and undetectable below 0.45 M OFHD, at which Figure 4 shows the α-helix conformational transition to be nearly complete.

Plots of emission maximum versus excitation wavelength for unbuffered, pH 2.0 solutions containing 1.6 M HFPD and 3.1 M TFBD begin at 353−354 nm, just as does Figure 6b. Also, over the 280−309 nm excitation wavelength range, the plots show a REES the same as in Figure 6a for a melittin solution containing no OFHD. So HFPD and TFBD show no detectable net REES.

In summary, the small to nonexistant net REES for melittin in solutions containing fluorinated diols suggests that motion of these molecules is not as restricted as is the motion of DOPC molecules in vesicles when they envelop melittin. Thus, it can be concluded that any clusters that may be formed by the fluorinated diols are not as organized as are the vesicular DOPC clusters.

OFHD as One of the Most Effective Helix Inducers. The C_m values for induction of helix formation in both melittin and β-lactoglobulin A by OFHD are lower than the corresponding values reported for HFIP.[26] As such, these are the lowest values of C_m for helix induction by alcohols in these polypeptides of which we are aware. Even when concentrations based on mass are considered, OFHD is more effective than HFIP. That is, for HFIP and OFHD, C_m for helix formation in β-lactoglobulin A corresponds to 110 and 81 g/L, respectively, and 69 and 58 g/L for helix formation in melittin, respectively. These results suggest the possible use of OFHD for applications that require the lowest possible concentrations of reagents to induce helix formation in other proteins.

Conclusions

Helix induction in melittin and β-lactoglobulin A is enhanced by fluorinated diols with the order of efficiency TFBD < HFPD < OFHD. The trend of increasing efficiency with increasing number of fluorines is consistent with results for fluorinated monoalcohols.[27] The helix-induction potential for OFHD is the greatest for any fluorinated alcohol of which the authors are aware. The potential for helix induction increases exponentially with the number of fluorine atoms in the alcohol molecule. The smooth scaling of *m* values for fluorinated diols and monoalcohols shows that the helix-inducing potential is about the same for fluorine atoms bonded to interior or exterior carbon atoms. The symmetric placement of hydroxyl groups on the ends of the fluorinated diol molecules prevents them from forming micelle-like assemblies and excludes them from being responsible for the mechanism associated with the conformational transition. The small to absent red-edge excitation shift of melittin fluorescence shows that the presence of organized aggregates with internal molecular mobility comparable to that of vesicles is not essential for helix induction by fluorinated diols.

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