Structure and Solvation of Melittin in 1,1,1,3,3,3-Hexafluoro-2-Propanol/Water

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ABSTRACT Fluorinated alcohols can induce peptides and proteins to take up helical conformations. Nuclear Overhauser effect (NOE) spectroscopy experiments and analysis of $C_\alpha H$ proton chemical shifts show that the conformation of melittin in 35% hexafluoro-2-propanol/water is α-helical from residues Ile-2 to Val-8 and from Leu-13 to Gln-25. As has been found in other solvent systems, the two helical regions are not colinear; the interhelix angle ($73 \pm 15^\circ$) in 35% 1,1,1,3,3,3-hexafluoro-2-propanol/water is smaller than the angle found in other fluoroalcohol-water mixtures or in the crystal. Intermolecular $^1H\{^{19}F\}$ and $^1H\{^1H\}$ nuclear Overhauser effects were used to explore interaction of solvent components with melittin dissolved in this solvent mixture. The NOEs observed indicate that fluoroalcohol and water molecules are both tightly bound to the peptide in the vicinity of the interhelix bend. For the remainder of the molecule, solute-solvent NOEs are consistent with preferential solvation of the peptide by the fluoroalcohol component of the solvent mixture.

INTRODUCTION

It has long been known that the addition of fluoroalcohols to aqueous solutions of peptides can induce formation of helical conformations. Many experimental and computational studies have attempted to elucidate the reasons for this effect. Most such efforts have focused on trifluoroethanol (TFE)-water mixtures (Buck, 1998) although more highly fluorinated alcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and hexafluoroacetone hydrate typically are more potent in producing conformational effects (Hirota et al., 1997). There is no single agreed-upon mechanism that accounts for the effects of fluorinated alcohols on peptide conformation (Buck, 1998). Among consideration likely to be important are changes in hydrogen bonding patterns and strengths (Cammers-Goodwin et al., 1996; Luo and Baldwin, 1997; Plass et al., 1995; Rothemund et al., 1996), enthalpic and entropic effects (Andersen et al., 1999), the physical properties of fluoroalcohol-water mixtures (Cammers-Goodwin et al., 1996; Walgers et al., 1998), and alteration of the kinetics of conformational changes (Cammers-Goodwin et al., 1996; Hong et al., 1999). Recent experiments and computer simulations indicate that preferential interaction of a peptide with the fluoroalcohol component of a fluoroalcohol-water mixture takes place in these systems (Bodkin and Goodfellow, 1996; Rajan et al., 1997; Diaz and Berger, 2001; Diaz et al., 2002; Gast et al., 2001; Fiorini et al., 2002) and such ''solvent sorting'' may be an important aspect of the conformational effects produced by fluoroalcohols.

Intermolecular NOEs can result from interactions between solute and solvent molecules. When solute-solvent interactions are weak, observed intermolecular dipolar effects can be understood in terms of random collisions of solute and solvent molecules. The NOE then depends on mutual diffusion of the interacting species and their distance of closest approach (Hennel and Klinowski, 1993; Noggle and Schirmer, 1971). However, interactions between the solute and solvent may be strong enough that the rotational and translational motions of both species are affected. In the limit of a very strong attractive interaction, solvent molecules in effect become part of the solute and move with it. In this limit, dipole-dipole interactions between solvent spins and solute spins are modulated by the dynamics of the complex, rather than by encounters of the solvent and solute molecules. In weaker interactions with a slowly tumbling (macromolecular) solute, the NOE is dominated by the dynamics of the solute even when there is substantial motion of the solvent molecule within the interaction complex, provided the solvent molecule remains within about one molecular diameter of the solute for a time long compared to the rotational correlation time (Otting, 1997). Otting has given a thorough summary of these limiting cases in the context of water molecule interactions with biological structures (Otting, 1997).

We began this study of the peptide melittin, a principal component of the venom of *Apis mellifera*, to explore the abilities of solvent-solute intermolecular nuclear Overhauser effects to report on peptide conformation. Calculations using the methods described below confirm one's intuition that intermolecular solvent-solute NOEs in the weak interaction limit should reflect local secondary structure of a peptide. For example, interactions of solvent molecules with amide (N-H) protons of a peptide backbone are expected to be from three- to fourfold smaller when the peptide is in an α-helix compared to a β-sheet structure. Previous work has shown that melittin is monomeric and helical at pH 2 in HFIP-water mixtures with fluoroalcohol concentrations as low as ~1 M (Hirota et al., 1997). We have determined the conformation of melittin in 35% HFIP (3.3 M) by standard methods based

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on intramolecular $^1H\{^1H\}$ NOEs. Intermolecular $^1H\{^{19}F\}$ NOEs produced by interactions between fluorine atoms of the solvent and peptide protons, and $^1H\{^1H\}$ intermolecular NOEs resulting from interaction of peptide protons with water in the solvent have been measured. Our observations confirm that the peptide conformation is largely helical in 35% HFIP/water and provide evidence that the components of the solvent mixture interact specifically and strongly enough with parts of the peptide such that a weak solute-solvent interaction model is not an appropriate description of these interactions. In agreement with the conclusions of others regarding smaller peptides dissolved in fluoroalcohol-water mixtures, our results show that water is largely excluded from the vicinity of the peptide in 35% HFIP/water.

MATERIALS AND METHODS

Melittin (>85% by HPLC) and 1,1,1,3,3,3-hexafluoro-2-propanol-d2 were obtained from Sigma-Aldrich (St. Louis, MO). Distilled, deionized water was used for sample preparation.

All NMR spectra were run at a proton frequency of 500 MHz using a Varian INOVA instrument. A Nalorac proton-fluorine probe with a z axis gradient capability was used. Sample temperatures were regulated by the instrument controller and were calibrated using a standard sample of methanol (Wilmad, Buena, NJ). Temperatures are believed to have been stable to better than $\pm 0.1°$ and accurate to better than $\pm 0.5°$. Field gradients were calibrated as described previously (Strickler and Gerig, 2002). Samples for NMR experiments were ~3.1 mM in solute. The pH of the sample, uncorrected for deuterium in the solvent mixture, was 1.99 as determined by model IQ150 pH meter (IQ Scientific Instruments, San Diego, CA) equipped with a 4-mm outer diameter stainless steel electrode. Samples were degassed by several freeze-thaw cycles before being sealed under vacuo in 5 mm J. Young tubes (Wilmad). A thin sealed capillary containing acetone-d6 for locking purposes was included in the sample.

Proton and fluorine T_1 relaxation times were determined by inversion-recovery and saturation-recovery methods to check for the possible influence of radiation damping. Data workup for these experiments used signal integration; any difference between the results of the two types of T_1 determination would indicate complications arising from radiation damping effects (Mao and Guo, 1994; Mao and Ye, 1997). Results of two methods typically agreed to within better than 1%, suggesting that radiation damping effects were negligible under our experimental conditions.

Pulse sequences used for total correlation spectroscopy (TOCSY) and NOE spectroscopy (NOESY) experiments were based on those of Fulton and Ni (1997). TOCSY mixing times were typically 30 or 70 ms whereas NOESY mixing times ranged from 100 to 300 ms. The pulse sequence and general methods used for $^1H\{^{19}F\}$ heteronuclear NOE experiments were the same as used previously (Gerig, 2003). Selective homonuclear NOE and ROE spectra were collected using pulse sequences based on those of Dalvit (1998). Gaussian pulses were used for selective inversion of the water signal (Emsley and Bodenhausen, 1990). Because the water signal is quite broad in these samples (~30 Hz) inversion by selective pulses is not efficient; the extent of inversion achieved in the experiments was determined and the appropriate corrections applied. Rotating frame NOE enhancement spectroscopy (ROESY) experiments with melittin in 35% HFIP/water were difficult because of rapid transverse relaxation and mixing times had to be <50 ms to obtain useful results.

The backbone N-H resonances of melittin in 35% HFIP/water were broad enough (up to ~6 Hz) that it was not possible to obtain $^3J_{NH\text{-}C\alpha H}$ coupling constants by direct observation. Attempts to obtain these data by spin echo methods were not successful due to the rapid T_2 relaxation of these signals.

The cross-relaxation rate constant (σ_{HX}) characteristic of the interaction of solvent spins X with a proton of the solute was estimated as described previously (Gerig, 2003). Plots of solute signal intensity data as a function of mixing time were analyzed to obtain their initial slopes that were taken to define σ_{HX}. Probable errors for the derived σ_{HX} depend on the separation of a signal of interest from others as well as the signal/noise ratio of the data. It is estimated that the reliability of the σ_{HX} values reported ranges from ± 10 to 20%.

Diffusion coefficients for solute and solvent components were determined by the method of Wu et al. (1995) using proton and fluorine signals of the sample. A weak gradient was present during the mixing time. A DPFGSE sequence was appended at the end of the basic pulse sequence for suppression of the solvent water signal in proton-observe experiments (Hwang and Shaka, 1995). Melittin diffusion coefficients were determined using proton NMR signals of the solute methyl groups. Samples were equilibrated in the NMR probe for several hours before diffusion experiments were started to minimize the effects of thermal gradients. Experiments were then run repetitively until three successive determinations of the diffusion coefficient agreed to within ~1%.

NOESY data were analyzed using the program SPARKY (Goddard and Kneller, 2004). About six hundred crosspeaks were assigned. These led to 229 unique, conformation-sensitive internuclear distances between hydrogens of residues separated by up to four amino acids. Further information on the observed NOEs is given in the supplementary material. The program DYANA was used to find conformations consistent with the assigned $^1H\{^1H\}$ NOEs (Guntert et al., 1997). The 10 best structures defined by DYANA were then relaxed in the AMBER 4.1 force field with the same distance constraints using SYBYL (Tripos). The energy penalty function for violation of an NOE constraint was $E_{NOE} = k(d - d_0)^2$ where d_0 is the upper or lower bound for a particular H-H distance, d is this distance in a particular conformation, and the constant $k = 25$ kcal/mol Å^2 (Case and Wright, 1993).

Molecular modeling was done using SYBYL (Tripos, St. Louis, MO) and the AMBER 4.1 or MMPF force field. Structure drawings and computation of the angle between the helical segments of melittin were done using the facilities of MOLMOL (Koradi et al., 1996).

Estimations of solvent spin-solute spin intermolecular NOEs in the weak interaction limit were done by the numerical procedure described previously (Gerig, 2003; Strickler and Gerig, 2002). Solvent molecules were represented by spheres. The effective molecular radius of the solvent sphere was estimated by the following method. A molecular model of the solvent molecule was constructed using standard bond lengths and angles. After minimization of the conformational energy, a van der Waals surface for the model was calculated using the Connolly method (Connolly, 1983). The radius of the sphere ''rolled'' over the surface of the model in this calculation was 1.2 Å, taken to be the van der Waals radius of a covalent hydrogen atom. Distances from the points representing the surface to the central atom of the solvent molecule were calculated and averaged. Using this approach it was estimated that the average radius of HFIP and of water are 2.79 and 1.66 Å, respectively. (Considering the pure liquids as being composed of cubic closest packed spheres leads to sphere radii of 3.14 and 1.74 Å, respectively.) When calculating dipolar interactions, it was assumed that all fluorine or hydrogen atoms of a solvent molecule are located at the center of the solvent molecule (Otting et al., 1997).

RESULTS

Structure of melittin in 35% HFIP/water

The proton NMR spectrum of melittin in the HFIP-water mixture used for this work was generally similar to the spectrum of the peptide in 50% hexafluoroacetone hydrate/water reported by Bhattacharjya et al. (1999) and assignment of the spectrum was appreciably aided by their efforts. Fig. 1 shows deviations of the observed $C_\alpha H$ proton chemical shifts from the random coil values given by Wishart and Sykes

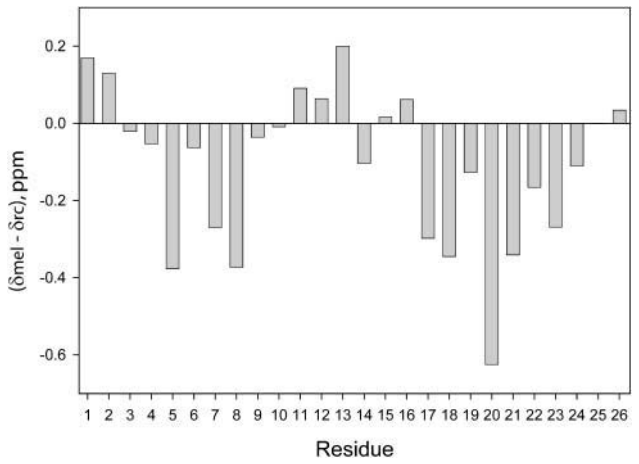

FIGURE 1 Deviations of the C_α-H proton chemical shifts from random coil values observed for melittin in 35% HFIP/water, 25°, and pH 2. Random coil shifts are from Wishart and Sykes (1994) and were corrected for sequence dependence according to the prescription of Schwarzinger et al. (2001).

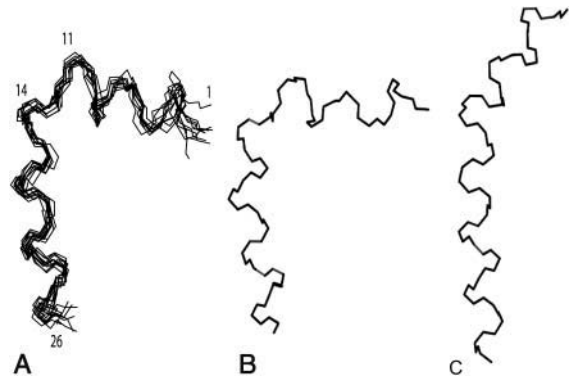

FIGURE 2 α-Carbon backbone plots of (*A*) 10 low-energy conformations found by analysis of NOESY data, (*B*) the mean conformation of melittin found in this work, and (*C*) the conformation of melittin observed in the crystalline state (chain *A*). See the supplementary material for a different rendering of the 10 low-energy conformations.

(1994), corrected for sequence dependence (Schwarzinger et al., 2001). This comparison may not be completely valid because the random coil shifts used have not been corrected for dependencies of the shifts on the mixed organic/water solvent system used. However, high concentrations of trifluoroethanol appear to have minimal effects on C_αH chemical shifts up to 50% TFE (Merutka et al., 1995) and we presume that a similar situation obtains in 35% HFIP. Appreciable upfield shifts of C_αH are expected for residues within helical conformations. Thus, the chemical shift data in Fig. 1 suggest that residues from Gly-3 to Thr-10 and from Ile-17 to Gln-26 probably are in helical conformations.

The tertiary structure of melittin in 35% HFIP/water was accessed through analysis of intramolecular $^1H\{^1H\}$ NOEs. Calculations using distances indicated by these NOEs as conformational constraints produced a collection of low-energy conformations consistent with the available data (Fig. 2). Most constraints were satisfied, although 18 distance limits were violated by >0.1 Å (see Supplementary Material). Ten low-energy conformations were collected. After conformational energy minimization in the AMBER 4.1 force field, the root mean square deviation of backbone atoms of these from the mean was ±1.3 Å whereas the root mean square deviation of the heavy atoms of the side chains was ±2.1 Å. An analysis of these structures by the program PROCHECK showed that all residues were in most favored (81%) or allowed regions (19%) of (ϕ,ψ) space (Laskowski et al., 1996). A secondary structure analysis by the program MOLMOL identified residues 2–8 and residues 13–25 as being in α-helical conformations, consistent with the implications of the C_αH shift analysis indicated earlier.

Fig. 2 also shows the conformation of melittin in crystals formed from aqueous solution (Terwilliger and Eisenberg, 1982). Two distinct helical regions are observed in the crystalline state, with an angle of 126° between the helix axes. In 35% HFIP/water the NMR data indicate that the two helical regions are preserved but are oriented at 73 ± 15° where the uncertainty given is the average deviation of the interhelix angle from the mean of the 10 NOE-derived structures analyzed. The two helical segments were found to be essentially coplanar in most but not all of these structures.

HFIP-melittin $^1H\{^{19}F\}$ intermolecular NOEs

Cross-relaxation rates (σ_{HF}) arising from dipolar interactions between the hydrogens of the solute and fluorines of the HFIP in the solvent mixture were determined. Provided that the interactions between solute and solvent are weak enough that they depend only on diffusive encounters of solute and solvent, the cross-relaxation rate σ_{HF} can be estimated by a numerical integration procedure based on the work of Ayant et al. (1977) and Gerig (2003). That procedure assumes that a solvent molecule can be represented by a sphere of radius r_s whereas a hydrogen of melittin is regarded as a sphere with a radius equal to its van der Waals radius, taken to be 1.2 Å in this work. The diffusion constants of the peptide and HFIP needed for the calculations were determined to be 7.12×10^{-9} m^2s^{-1} and 5.21×10^{-8} m^2s^{-1}, respectively.

$^1H\{^{19}F\}$ NOEs for many protons of melittin dissolved in 35% HFIP/water were reasonably well predicted by the numerical integration procedure. Signals for the methyl groups of the Thr-10 and Thr-11 side chains are readily distinguished in the ^1H NMR spectrum; experimental values of σ_{HF} for these are 6.3×10^{-3} and 6.2×10^{-3}, respectively, whereas the calculated values are $6.4 \pm 0.7 \times 10^{-3}$ and $6.5 \pm 0.4 \times 10^{-3}$. (The calculated σ_{HF} are averages for the 10 NMR structures shown in Fig. 2 *A*; the uncertainties are the standard deviations.) Fig. 3 compares observed and calculated values of σ_{HF} for hydrogens of Trp-19; most calculated and observed σ_{HF} values for this residue

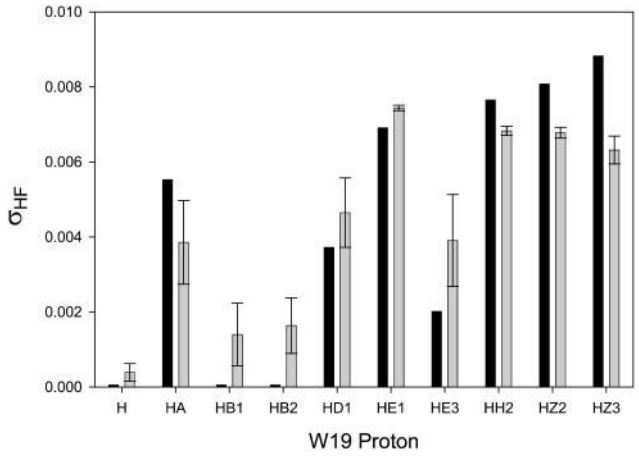

FIGURE 3 Comparison of observed σ_{HF} (*solid bars*) and calculated σ_{HF} (*shaded bars*) for the hydrogens of the Trp-19 residue of melittin. The calculated data are the average of calculations for the 10 conformations found in the analysis of intramolecular NOE data (Fig. 2); the error bars represent the standard deviation of these data.

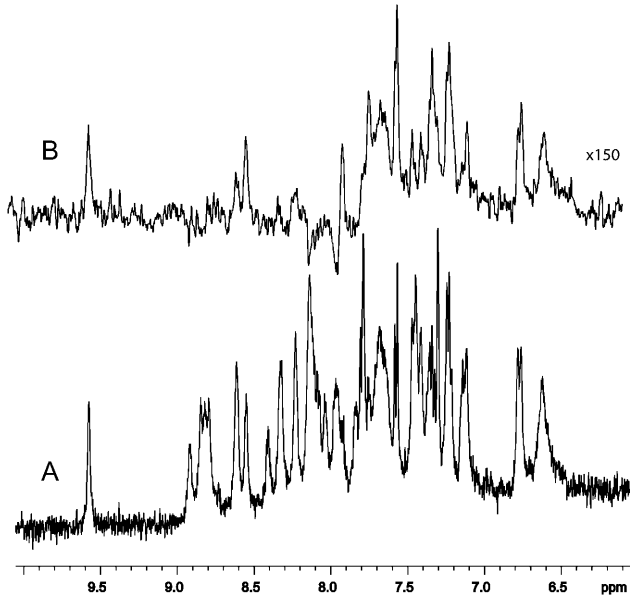

FIGURE 4 (*A*) Low field proton NMR spectrum of melittin in 35% HFIP/water and (*B*) result of an intermolecular $^1H\{^{19}F\}$ NOE experiment with a mixing time of 800 ms. Spectrum *A* is due to a single scan whereas spectrum *B* is a difference spectrum arising by subtracting 6000 control spectra from 6000 scans containing the NOE.

agree when experimental uncertainties and variations between the NMR structures are considered. Signals for methyl groups of isoleucine, leucine, and valine side chains are overlapped in the 0.8–1.1 ppm region of the proton spectrum of the peptide. Experimental values for σ_{HF} in this part of the spectrum range from 7 to 9 $\times$ 10^{-3} whereas calculations predict σ_{HF} lies between 3 and 6 $\times$ 10^{-3}. There is some uncertainty in the computational results for methyl groups because the procedure used does not recognize the internal dynamics of these groups. Overall, and bearing in mind the rather large experimental errors associated with σ_{HF}, it appears that the procedures and assumptions used to estimate σ_{HF} (random collisions of HFIP and melittin and weak solute-solvent interactions) give reasonably reliable results, at least for side chain-solvent interactions. Thus, there are no indications of especially strong, presumably hydrophobic, interactions between melittin side chains and the fluoroalcohol component of the solvent.

Fig. 4 shows the low field region of the proton NMR spectrum of melittin in 35% HFIP/water. Also indicated in the figure are the $^1H\{^{19}F\}$ NOEs observed for the signals in this spectral region. Fig. 5 compares observed σ_{HF} for the N-H protons of melittin to σ_{HF} calculated by the procedures indicated previously. Low signal/noise ratios or peak overlaps made it difficult to determine weak NOEs reliably and some or all of the experimental σ_{HF} indicated as being zero in Fig. 5 could actually be small negative or positive values. However, it is apparent that for peptide N-H protons, the expected dependence of σ_{HF} on conformation is generally observed. Major exceptions are at the NH signals for Gly-12, Leu-13, Leu-16, and Ser-18 where negative values of σ_{HF} are observed whereas small positive values are predicted. Signals for Gly-12, Leu-13, and Ser-18 are overlapped enough that separate values of σ_{HF} for these could not be determined. We will refer to these hydrogens as a group in the discussion that follows, while recognizing that it is possible that the magnitudes of some σ_{HF} values for these three residues are small whereas the remainder is strongly negative.

No reasonable set of the parameters used in the predictions of σ_{HF} based on the random collisions model could be found that produced negative values of σ_{HF}. However, we note that the rotational correlation time of melittin in water is ~1.5 ns (Kemple et al., 1997; Zhu et al., 1998). Relaxation data suggest that the viscosity of 35% HFIP/water is higher than pure water (Yoshida et al., 2003) and a rotational correlation time for melittin of ~3 ns is estimated for our system, a value consistent with the observed translational diffusion coefficient of monomeric melittin if it is assumed that the peptide can be represented by a sphere of radius 10–14 Å. Calculations show that a negative σ_{HF} would be expected for H-F dipolar interactions if a HFIP molecule interacts strongly enough with melittin that it takes on the rotational characteristics of the peptide. As an exercise, SYBYL was used to create a molecular model of the peptide surrounded by a solvent shell of HFIP. Consistent with MD simulations of this system (Fiorini et al., 2001), this model showed that fluorine atoms could be within ~4.5 and ~6.5 Å of the backbone NH atoms of Gly-12–Ser-18. Assuming that fluoroalcohol molecules are firmly enough bound at these distances that their rotational correlation time is 3 ns, σ_{HF} would be between −0.002 and −0.014 for each H-F interaction. The cross-relaxation rate would still be negative if an interacting HFIP molecule rotates rapidly but remains

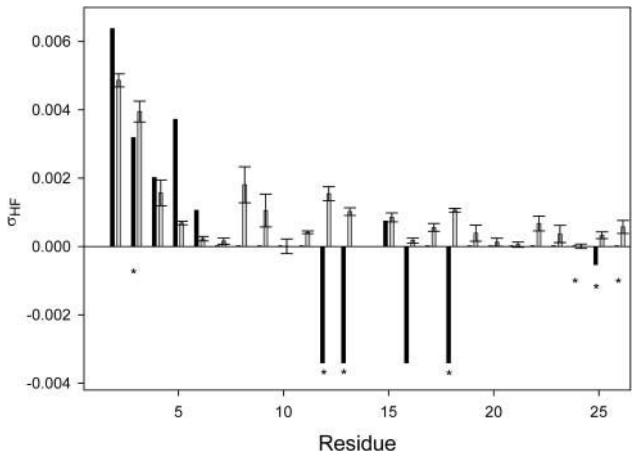

FIGURE 5 Comparisons of observed (*solid bars*) and calculated (*shaded bars*) values of σ_{HF} of the peptide backbone N-H signals in melittin in 35% HFIP/water. Error bars represent standard deviations of calculated data for the 10 low-energy conformations found in the structure determination. Asterisks indicate ambiguous experimental results. The signals for Gly-12, Leu-13, and Ser-18 are overlapped; one or more of the experimental NOEs for any of these could be zero. The signals for Gly-3 and Arg-24 are overlapped; based on the calculations it has been assumed that the observed NOE signal arises from Gly-3. The signal for Gln-25 could be influenced by the nearby signals of Gly-12, Leu-13, and Ser-18. Experimental values of σ_{HF} indistinguishable from 0 have been set to 2×10^{-5} in the plot to provide indications of the positions of these data.

associated with the peptide (Otting, 1997). We do not know the details of the dynamics of strongly interacting HFIP in a solvent-melittin complex. However, the observation of negative σ_{HF} values suggests that one or more HFIP molecules are strongly enough interacting in the vicinity of the backbone NH protons of Gly-12, Leu-13, Leu-16, and Ser-18 that motions of these fluoroalcohol molecules are significantly influenced by the motion of the peptide.

Attempts were made to estimate σ_{HF} for the $C_\alpha H$ protons of the peptide backbone. These efforts were frustrated by overlaps of these signals; the data collected are shown in the supplementary material. Comparisons of the available experimental to calculated values of σ_{HF} obtained generally indicated that experimental σ_{HF} values for $C_\alpha H$-HFIP interactions were appreciably smaller (by a factor of $\sim$2) than the predicted values. It seems hazardous to draw conclusions from these data until the problem of clean identification of NOEs at specific $C_\alpha H$ protons is addressed. However, a smaller (less positive) than expected σ_{HF} at any of these positions could also be indicative of strong interactions with HFIP molecules.

Water-melittin $^1H\{^1H\}$ NOEs in 35% HFIP/water

Cross-relaxation characterized by the rate σ_{HH} could be the result of diffusive encounters between solvent water molecules and the hydrogens of the peptide, but could also arise because solvent protons exchange with acidic protons of the peptide (Otting, 1997). Direct chemical exchange in which a proton of melittin is replaced by a solvent proton may be diagnosed by comparison of ROESY and NOESY data (Neuhaus and Williamson, 2000). Table 1 records observed σ_{HH} values that were not the result of direct exchange. All of these NOEs were also observed in rotating frame NOE enhancement (ROE) experiments although the ROE experiments were not analyzed values for σ_{HH}. It is striking that so few side-chain proton-water proton NOEs are observed. There are no indications of water proton NOEs with the carbon-bound ring protons of the Trp-19 side chain even though the $H_{\varepsilon 1}$ proton undergoes exchange with water.

Calculations of intermolecular water proton-melittin proton σ_{HH} values for the random encounter model were carried out using the same procedures as described earlier. A separate signal for the HFIP OH proton was not observed so that exchange of this proton and water protons in the solvent mixture is at least in the intermediate exchange regime. Perturbation of the ''water'' line of the spectrum thus inverts both the water and HFIP hydroxyl spins. HFIP molecules that interact with the peptide will produce a contribution to the apparent water cross-relaxation rate. However, the concentration of water protons in the solvent mixture is 72 M whereas the concentration of HFIP hydroxyl protons is 3.3 M. Thus, even if the dipolar interactions of the HFIP hydroxyl protons are ignored, an error of $<5\%$ arises.

That these procedures give reasonably reliable predictions of σ_{HH} for diffusive encounters of weakly interacting solute and solvent species is indicated by σ_{HH} for an impurity peak in the spectrum at 2.21 ppm. The material responsible for this peak has not been identified but if it is assumed to be acetone or acetate, one estimates the diffusive contribution to σ_{HH} to be $+0.036$ using the experimental diffusion coefficients of this species and water, 6.3×10^{-8} m^2 $s^{-1}$and 1.2×10^{-7} m^2 s^{-1}, respectively. This result is in reasonably good agreement with the experimental observation, $\sigma_{HH} = +0.025$. All experimental solute proton-water proton NOEs observed for melittin in 35% HFIP/water are characterized by negative values of σ_{HH} and, thus, cannot be simply the result of diffusive encounters between water molecules and the peptide.

TABLE 1 $^1H\{^1H\}$ melittin-water NOEs

Shift (ppm)	Assignment	σ_{HH} (calculated)	σ_{HH} (observed)
1.02	Leu-6 H^δ, Leu-9 H^δ	0.031–0.033	−0.019
1.09	Leu-13 H^δ, Leu-16 H^δ, Ile-17 H^γ,	0.030–0.032	−0.011
1.51	Thr-11 H^γ	0.034	−0.32
1.54	Thr-10 H^γ	0.034	−0.40
1.66	Ile-2 H^γ, Leu-6 H^γ, Lys-7 H^γ, Ala-4 H^β	0.033	−0.075
1.84	Lys-7 H^δ, Leu-16 H^β, Ile-17 H^γ, Lys-21 H^δ	0.029–0.033	−0.050
3.11	Lys-21 H^ε, Lys-23 H^ε	0.028–0.033	−0.067
8.14	Gly-12 NH, Leu-13 NH, Ser-18 NH	0.014–0.016	−0.12
8.33	Thr-10 NH, W-19 NH	0.007–0.009	−0.075

Negative values of σ_{HH} not due direct exchange were found for the N-H protons of Gly-12, Leu-13, and Ser-18 (overlapped) and Thr-10 and Trp-19 (overlapped). Many of the observed NOEs are for carbon-bound hydrogens on side chains that have polar groups capable of hydrogen bonding or exchange with protons of the solvent (Lys, Thr). Chemical exchange of solvent protons with the acidic protons of these side chains probably leads to the observed NOEs, although it cannot be ruled out that interactions between solvent water molecules and these polar parts of melittin are sufficiently strong that water molecules may become involved in what are essentially intramolecular dipolar interactions, similar to the situation involving strong HFIP interactions that was discussed earlier. Interestingly, small negative values of σ_{HH} are also observed for the methyl groups of leucine and isoleucine side chains.

DISCUSSION

Intramolecular $^1H\{^1H\}$ NOE data indicate that the structure of melittin in 35% HFIP/water consists of two helical regions (roughly residues Ile-2–Val-8 and Leu-13–Gln-25) with a transition region containing residues Leu-6–Pro-14 consisting of bend and turn local conformations. Our analysis of the $^1H\{^1H\}$ NOEs indicates that the helical regions are on average oriented at 73 ± 15° relative to each other (Fig. 2). The residues in the vicinity of the bend are not found with canonical α-helical conformational angles in all structures produced in the NOE analysis nor are the two helices always precisely in a common plane. It thus appears likely that the transition region between the two helices is fairly flexible when compared to the rest of the structure. The reported mean residue molar ellipticity at 222 nm of melittin in 35% HFIP/water and 20° indicates that the peptide is ~70% helical (Hirota et al., 1997), a conclusion in agreement with our structure. It has been observed that the interhelix angle of melittin depends on the environment of the peptide, with reported values for this angle ranging from 86 ± 34° when the peptide is contained in vesicles of phosphatidylcholine (Okada et al., 1994) to 160° in methanol (Bazzo et al., 1988). Bhattacharjya et al. (1999) have determined the conformation of melittin in 50% hexafluoroacetone hydrate/water, finding a structure that has ''a well-ordered helical fold with a relatively flexible segment'' near residues Thr-10–Gly-12. They concluded that the averaged NMR structure under their conditions is less distorted in the flexible segment than is the case in the crystal structure (Terwilliger and Eisenberg, 1982). The molar concentrations of covalent fluorine in 35% HFIP/water and 50% hexafluoroacetone hydrate/water are virtually the same (~21.5 M) and, if fluorine-solute interactions are primarily responsible for the formation of helical conformations in these fluoroalcohol-water mixtures, it is surprising that the conformations detected by NMR in 35% HFIP/water and 50% hexafluoroacetone hydrate/water are not more similar.

It is important that we be able to obtain a reliable idea regarding the sign and magnitude of intermolecular solute-solvent NOEs in the absence of strong interactions because it is deviations from these expectations that indicate unusual solvent interactions with dissolved melittin. The computational procedures used to estimate intermolecular NOEs due to random peptide-solvent collisions have been tested in systems where the weak interaction limit should be a good approximation (Gerig, 2003). The assumptions behind the calculations include: 1), that solvent molecules are approximately spherical and 2), that interactions between solvent and solute are so weak that their encounters are described by simple diffusion. When these assumptions are reasonably adhered to, the reliability of computed predictions of the cross-relaxation rate σ_{HX} probably is of the order of ±20% (Gerig, 2003).

A complete study of solvent interactions with all protons of melittin in 35% HFIP/water is impossible by the one-dimensional NMR techniques used in this work because of signal overlaps. However, it is clear from the available data that most side-chain protons, backbone N-H, and C_α-H protons of the peptide interact with the HFIP component of the solvent mixture essentially as expected from encounters that are mediated only by the mutual diffusion of the peptide and HFIP. However, intermolecular NOEs between fluorines of HFIP and some backbone protons in the transition region between the helices are negative, plausibly due to the formation of long-lived, solvent-peptide complexes. These interactions could also impact NOEs for side-chain protons of the residues in the transition region as well. A small negative σ_{HF} was tentatively identified for Leu-9 H^β and Leu-13 H^β but, unfortunately, most solvent NOEs to side chains of residues 6–14 could not be determined reliably from our data due to overlaps of signals. However, no strongly negative NOEs beyond those noted were apparent for any other signals observed in the spectrum. It may be that positive values of σ_{HF} expected for many protons as a result of diffusive encounters are counterbalanced by negative contributions from locally restricted HFIP molecules so that the resulting effect is small.

Simple diffusive encounters are not consistent with the observed intermolecular NOEs between the water molecules of the solvent mixture and protons of the peptide. Comparison of NOESY and ROESY data indicates that the negative $^1H\{^1H\}$ water NOEs observed with some or all the N-H protons of the Gly-12, Leu-13, Ser-18 group at 7.98 ppm and the N-H protons of the Thr-10 and/or W-19 at 8.17 ppm most likely arise from water molecules that are immobilized within the conformational transition region of the peptide.

Negative $^1H\{^1H\}$ NOEs are also observed for methyl groups of leucine and isoleucine. Leu-6, Leu-9, and Leu-13 are in the conformational transition region and any or all of these side chains could be influenced by water molecules interacting strongly with the peptide in this part of its structure. A conclusion consistent with all of the solute-

solvent NOE observations that we report is that both HFIP and water molecules interact strongly enough with the peptide backbone of melittin between Thr-10 and Leu-16 and the side chains of leucines so that the motion of the solvent molecules are influenced by the rotational tumbling of the peptide. It will not be possible to be more precise about the location or dynamics of these relatively immobilized solvent molecules until more NOEs of individual protons in this part of the melittin structure become available. However, our results show that both components of the fluoroalcohol-water solvent mixture interact more strongly with the amino acids located in the transition region between the helices of the peptide than with the amino acids that are located in the α-helical regions.

Although intermolecular NOEs provide ample evidence of fluoroalcohol interactions with the protons of melittin, beyond the NOEs noted in Table 1 there is little evidence for interactions of water molecules with the peptide. Our results are thus consistent with qualitative observations of the interactions of trifluoroethanol-water mixtures with peptides reported by Berger's group (Diaz and Berger, 2001; Diaz et al., 2002). Using intermolecular NOE observations, these workers found that the fluoroalcohol component of the mixed solvent preferentially associated with several peptides, leading to fluorine-solute proton NOEs whereas water proton-solute proton NOEs were reduced or absent.

Preferential solvation of peptides by the fluoroalcohol component of fluoroalcohol-water mixtures have been observed in molecular simulations (Bodkin and Goodfellow, 1996; Brooks and Nilsson, 1993; Fiorini et al., 2001; Iovino et al., 2001; Roccatano et al., 2002). In particular, solvent sorting has been observed in simulations of melittin in 30% HFIP/water (Fiorini et al., 2001) and 30% TFE/water (Roccatano et al., 2002). In the latter case, the local concentration of TFE about amino acid side chains of the peptide was ~2 times higher than the nominal concentration.

Increase in local fluoroalcohol concentration at distances close to the surface of melittin is inconsistent with the model used for calculating intermolecular $^1H\{^{19}F\}$ NOEs due to diffusive encounters (Gerig, 2003). That model assumes that a given melittin proton is surrounded by a homogenous solvent mixture. If local concentrations of fluoroalcohol were higher than the bulk concentration then higher values of σ_{HF} would be expected. However, we find reasonable agreement between experimental values of σ_{HF} and those predicted using the assumption of a homogeneous mixture of fluoroalcohol and water. The answer to the apparent conundrum may lie in the fact that HFIP at a concentration of 35% in water is appreciably aggregated (Gast et al., 2001; Hong et al., 1999; Kurpin et al., 1995). Although aggregation of HFIP appears not to be a fundamental requirement for the production of conformational effects, it is likely at the concentration of HFIP used in this work that melittin is associated with micelles of the fluoroalcohol (Gast et al., 2001). Scattering data indicate that HFIP clusters are characterized by a radius of gyration of ~14 Å. (Gast et al., 2001; Hong et al., 1999). If a cluster of HFIP molecules can be regarded as spherical, these data indicate that the radius of the cluster would be ~18 Å (van Holde et al., 1998). Calculations given in the Appendix show that, if it can be assumed: 1), that a given hydrogen of the melittin structure is immersed in a cluster of HFIP molecules of radius 18 Å; 2), that water is excluded from the region nearest the peptide; 3), that the number of HFIP molecules per cm^3 decreases as one moves from the interior of the HFIP aggregate to its surface (suggested by Fig. 9 of Fiorini et al., 2001) as $1/r$, where r is the distance from the center of the sphere; 4), that the diffusive behavior of HFIP molecules is independent of HFIP concentration or aggregation state; and 5), that the number of HFIP molecules per cm^3 at the distance of closest approach between melittin and HFIP is about twice the concentration in the bulk solvent, then a cross-relaxation rate due to random collisions with HFIP molecules in the aggregate equivalent to that produced by a homogeneous fluoroalcohol-water mixture can be expected. Calculations given in the Appendix also show how exclusion of water molecules from an aggregate of HFIP is consistent with a reduction of σ_{HH}. Both calculations are, of course, crude in that they neglect the dynamic aspects of an HFIP cluster and make arbitrary assumptions about the ways in which the concentrations of HFIP and water vary as the distance from the proton of interest increases. However, they serve to demonstrate that our experimental observations of σ_{HF} and σ_{HH} can be consistent with the known properties of the melittin-HFIP-water system at the HFIP concentration that was used for this work. It would be of interest to determine HFIP-solute NOEs under conditions where aggregation is not so extensive.

Specific solvation of a dissolved peptide (''solvent sorting'') in fluoroalcohol-water mixtures may be an important contributor to the conformational effects these mixtures produce (Bodkin and Goodfellow, 1996; Gast et al., 2001; Hong et al., 1999; Rajan et al., 1997). Our results show that the strengths or lifetimes of the interactions of both solvent components of a fluoroalcohol-water mixture with melittin are not uniform throughout the peptide structure. The strength and timescale of these interactions may be facets that will have to be considered in reaching an understanding of conformational effects produced by fluoroalcohol-water mixtures.

SUMMARY

The conformation of the peptide melittin 35% HFIP/water is α-helical between residues Ile-2 and Val-8 and from Leu-13 to Gln-26, with the connecting region from Leu-9 to Pro-14 in various turn conformations and probably rather flexible. This conclusion is consonant with observed $C_\alpha H$ chemical shifts and the ellipticity at 222 nm. Observed $^1H\{^{19}F\}$ and $^1H\{^1H\}$ intermolecular solute-solvent NOEs are consistent with this structure if it is assumed that water molecules are

largely excluded from direct interactions with the peptide in the helical regions. NOEs indicate that both HFIP and water molecules interact with hydrogens in the part of the structure between the helical regions sufficiently strong so that negative cross-relaxation rates are developed. The observed $^1H\{^{19}F\}$ NOEs are consistent with the known aggregation of HFIP under the conditions of the experiments. It is not known whether the strong solvent interactions with the peptide in the region between the helices are responsible for the disordering of the structure in this region, or develop because the structure is disordered.

APPENDIX: CALCULATIONS SUGGESTING THAT OBSERVED CROSS-RELAXATION RATES (σ_{HX}) ARE CONSISTENT WITH AGGREGATED HFIP IN 35% HFIP/WATER

Assumptions

A HFIP aggregate is assumed to be spherical with a radius r_{edge} of 18 Å. It is assumed that a sphere representing a HFIP molecule can make a closest approach (r_{dca}) to a melittin proton of 3.99 Å (1.2 Å + 2.79 Å) whereas a water molecule has $r_{dca} = 2.86$ Å (1.2 Å + 1.66 Å). Diffusion coefficients of solute and solvent are assumed to be the same throughout the model and equal to the experimental value for the bulk sample. In a solvent that is homogenous

$$\sigma_{HX} = CN_0 \int_{r_{dca}}^{r_\infty} \frac{dr}{r^2} = CN_o \frac{1}{r_{dca}},$$

where C is a collection of constants including the gyromagnetic ratios of the interacting spins H and X and N_0 is the concentration of X spins in units of spins/cm^3 (Hennel and Klinowski, 1993).

At the distance of closest approach for HFIP indicated above, $\sigma_{HX} = 0.25\ C\ N_0$. The contribution to σ_{HX} for HFIP in the homogenous solvent between r_{dca} and r_{edge} is

$$\sigma_{HX} = CN_0 \int_{r_{dca}}^{r_{edge}} \frac{dr}{r^2} = CN_0 \left(\frac{1}{r_{dca}} - \frac{1}{r_{edge}} \right).$$

For the assumed distances listed above, this contribution to σ_{HX} is 0.20 $C\ N_0$. Thus, for HFIP (and water) in the solvent mixture, σ_{HX} is largely determined by interactions within a sphere the same size as the aggregates formed by HFIP.

$^1H\{^{19}F\}$ NOEs

Assume that a solute proton is at the center of an aggregate of HFIP and that the concentration of fluorine (HFIP) spins, $N_F(r)$, varies from the distance of closest approach to the edge of the aggregate according to

$$N_F(r) = \frac{N_1 r_{dca}}{r},$$

where N_1 is a constant. It is assumed that the solute proton is accessible to the solvent equivalently from all directions and that, beyond the edge of the aggregate, the solvent is homogeneous with a fluorine spin concentration of N_0^F With these assumptions the contribution to σ_{HF} from the solvent molecules between r_{dca} and r_{edge} will be

$$\sigma_{HF} = C_F N_1 r_{dca} \int_{r_{dca}}^{r_{edge}} \frac{dr}{r^3} = C_F N_1 r_{dca} \left(\frac{1}{r_{dca}^2} - \frac{1}{r_{edge}^2} \right),$$

where C_F is a collection of constants. For the values of r_{dca} and r_{edge} indicated earlier

$$\sigma_{HF} = 0.119\, C_F N_1.$$

Thus, if the effect produced by fluorine spins of the solvent within the aggregate is equivalent to that produced by homogeneous solvent, it must be the case that in the aggregate $N_1 = 1.6\ N_0^F$, where N_0^F is the number of F spins/cc^3 in the homogeneous system. The concentration of these spins at the distance of closest approach in the aggregate is 1.6 N_0^F. Computer simulations of the HFIP-water system suggest the displacements of water molecules from a peptide surface by HFIP of this extent.

The assumption about the way the solvent spin concentration falls off with distance away from the target proton within the aggregate made in this calculation is reasonable, but completely arbitrary. However, other models for this concentration variation produce qualitatively similar results.

$^1H\{^1H\}$ NOEs

If the aggregate of HFIP surrounding a melittin proton of interest excludes water molecules from the vicinity of this proton, a reduction in σ_{HH} for dipolar interaction of water protons with solute protons is expected. If it is assumed that the concentration of water protons as one moves from the distance of closest approach to the edge of the fluoroalcohol aggregate is given by $N_H(r) = N_2\left(1 - \frac{r_{dca}}{r}\right)$, where N_2 is a constant and r is the distance of the solvent proton from the center of the ^{1}H nucleus of the solute, then the contribution of water protons to σ_{HH} from water-solute proton interactions between r_{dca} and r_{edge} is

$$\sigma_{HH} = C_H N_2 \int_{r_{dca}}^{r_{edge}} \frac{\left(1 - \frac{r_{dca}}{r}\right) dr}{r^2}$$

$$= C_H N_2 \left(\left(\frac{1}{r_{dca}} - \frac{1}{r_{edge}} \right) - \frac{r_{dca}}{2} \left(\frac{1}{r_{dca}^2} - \frac{1}{r_{edge}^2} \right) \right),$$

assuming that all solvent proton-solvent proton interactions are equivalent in all directions. Here the constants represented by C_H are different from those used for hydrogen-fluorine interactions considered above. If it is assumed that the concentration of water spins at the edge of the HFIP aggregate is the same as that of the bulk system (N_0^H) then $N_2 = N_0^H$ and σ_{HH} for water from r_{dcs} to r_{edge} will be 0.13 $C_H\ N_0^H$, ~43% of what would be predicted if the fluoroalcohol-water mixture were fully homogeneous.

Again, the choice of function to describe the water concentration in and about the HFIP aggregate is arbitrary, but the calculation serves to demonstrate that an aggregation of HFIP with exclusion of water molecules from the aggregate is consistent with the experimental observations reported.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

Assignments for melittin in 35% HFIP/water, a listing of the NOE-distance constraints used, plots indicating the distribution of the NOEs, an overlaid ribbon drawing of the peptide, and a plot of observed and calculated $C_\alpha H$ $^1H\{^{19}F\}$ NOEs are provided.

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