Biochimica et Biophysica Acta, 557 (1979) 135—148

BBA 78526

USE OF FULLY DEUTERATED MICELLES FOR CONFORMATIONAL STUDIES OF MEMBRANE PROTEINS BY HIGH RESOLUTION ^{1}H NUCLEAR MAGNETIC RESONANCE

LARRY R. BROWN

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule, 8093 Zürich-Hönggerberg (Switzerland)

(Received March 28th, 1979)

Key words: Membrane protein; ^{1}H-NMR; Deuterated micelles; Melittin

Summary

Micellar complexes of melittin with fully deuterated detergents have been studied by high resolution ^{1}H nuclear magnetic resonance (NMR). The synthesis of deuterated micelles is described and it is shown that the ^{1}H NMR spectrum of micelle-bound melittin is well resolved and suitable for detailed analysis by conventional high-resolution NMR methods. A preliminary characterization of micelle-bound melittin shows that interaction with the micelle results in different conformational and dynamic features for the hydrophobic and hydrophilic regions of the melittin amino acid sequence. The present experiments on melittin and preliminary results with other polypeptides and proteins demonstrate that in favourable cases high-resolution ^{1}H NMR studies of the complexes formed between membrane proteins and deuterated micelles provides a viable method for conformational studies of membrane-bound proteins.

Introduction

A major hindrance to the understanding of the functional properties of biological membranes is that only very limited information is available on the conformation of membrane-bound proteins. Furthermore, although the possible importance of protein-lipid interactions has been recognized, much of the extensive research into the structural basis of such interactions has been limited to study of lipid organization in the presence of bound proteins. Very little detailed information regarding lipid influence on protein conformation has as yet been obtained. The lack of such basic information for membrane-bound proteins is a consequence of the lack of suitable methods for analyzing protein conformation in the presence of associated lipids. For the two meth-

ods most extensively applied to detailed conformational characterizations of water-soluble proteins, namely X-ray diffraction and high-resolution nuclear magnetic resonance (NMR), the problem of associated lipids has previously prevented extensive application of these techniques to membrane-bound proteins. In view of the difficulty of obtaining three-dimensional crystals of membrane-bound proteins, high-resolution NMR may be a more promising approach for such proteins.

Previous work has shown that, with currently available methods, high-resolution ^1H NMR spectra suitable for detailed conformational analysis are not obtainable from proteins bound to native membranes [1,2] or from reconstituted systems consisting of proteins bound to phospholipid vesicles [3—5]. In the present paper, synthesis of fully deuterated micelles is described and it is shown that high-resolution ^1H NMR spectra suitable for detailed conformational analysis are obtained for melittin bound to micelles of fully deuterated detergents. To demonstrate the potential of this approach for conformational studies of membrane proteins, a preliminary characterization of the properties of melittin bound to fully deuterated dodecylphosphocholine micelles is presented.

Materials and Methods

Melittin was purified from lyophilized bee venom (H. Mack, Illertissen, F.R.G.) according to the method of King et al. [6]. Residual phospholipase A activity was removed by the method of Mollay et al. [7].

Fully deuterated dodecyl-(oxyethylene)$_8$-glycol was a gift from Dr. M. Chabre. Fully deuterated dodecylphosphocholine was synthesized as follows.

$$CH_3(CH_2)_{10}COOH \xrightarrow[PtO_2]{Na_2O_2,{}^2H_2O} C^2H_3(C^2H_2)_{10}COO^2H \xrightarrow[BF_3O(C_2H_5)_2]{NaB^2H_4} C^2H_3(C^2H_2)_{11}O^2H$$

$$\xrightarrow{(C_6H_5O)_2POCl} C^2H_3(C^2H_2)_{11}OPO(OC_6H_5)_2 \xrightarrow[H_2]{Pt/C} C^2H_3(C^2H_2)_{11}OPO_3H_2$$

$$HOC^2H_2C^2H_2Br + N(C^2H_3)_3 \rightarrow HOC^2H_2C^2H_2N^+(C^2H_3)_3Br^-$$

$$C^2H_3(C^2H_2)_{11}OPO_3H_2 + HOC^2H_2C^2H_2N^+(C^2H_3)_3CH_3COO^-$$

$$\rightarrow C^2H_3(C^2H_2)_{11}OPO_3^-C^2H_2C^2H_2N^+(C^2H_3)_3$$

[$^2H_{24}$]Dodecanoic acid. Dodecanoic acid (Fluka, greater than 99.5%) was deuterated by catalytic exchange with ^2H_2O according to Dinh-Nguyen et al. [8]. After two cycles of exchange, the reaction mixture was acidified with conc. H_2SO_4 and the deuterated fatty acid extracted into diethyl ether. After drying the product no impurities were detected by thin-layer chromatography. NMR showed that 98.7% exchange was achieved.

[$^2H_{26}$]Dodecanol. Without further purification, [$^2H_{24}$]dodecanoic acid was reduced by the procedure of Anhoury et al. [9]. Under a stream of dry nitrogen, 7.2 g (32 mmol) of [$^2H_{24}$]dodecanoic acid and 1.7 g (40 mmol) of NaB^2H_4 (Merck, Sharp and Dohme) were dissolved in 150 ml of freshly distilled tetrahydrofuran. 7.5 ml (60 mmol) of $BF_3O(C_2H_5)_2$ were added dropwise and the closed mixture stirred for 24 h. Addition of NaB^2H_4 and $BF_3O(C_2H_5)_2$

was repeated and stirring continued for a further 24 h. Absolute ethanol was added to destroy excess diborane, the bulk of the solvent removed by rotary evaporation, the mixture acidified with 4 N HCl, sufficient water added to dissolve all salts and the dodecanol extracted into diethyl ether. After washing and drying the diethyl ether phase, NMR and thin-layer chromatography showed the product to be dodecanol with slight traces of unidentified impurities. Nominal yield 5.9 g = 87%.

Diphenyl[$^2H_{25}$]dodecylphosphoric acid. The [$^2H_{26}$]dodecanol was phosphorylated without further purification using diphenylphosphoryl chloride [10]. 5.9 g (27.8 mmol) of slightly impure [$^2H_{26}$]dodecanol were dissolved in 70 ml dry pyridine and 7.3 ml (35 mmol) of diphenylphosphoryl chloride (Fluka) were added dropwise. The closed reaction vessel was stirred at 30°C overnight, a further 2 ml of diphenylphosphoryl chloride added and stirring continued for a further 12 h. 10 ml of H_2O was added to destroy excess diphenylphosphoryl chloride, the bulk of the pyridine removed by rotary evaporation and the oily residue taken up in diethyl ether. After washing the ether phase with 2 N H_2SO_4 and H_2O, the ether was removed to give a thick oil.

To remove impurities which interfered with removal of the phenyl groups by catalytic hydrogenation, this material was purified by column chromatography on silica gel 60 (Merck) using $CHCl_3/CH_3OH$ (9 : 1) as eluent. Removal of the $CHCl_3/CH_3OH$ from product-containing fractions gave a thick oil. No impurities were detectable by thin-layer chromatography or NMR. Yield 9.6 g = 78%.

[$^2H_{25}$]Dodecylphosphoric acid. The phenyl groups were removed from diphenyl[$^2H_{25}$]dodecylphosphoric acid by catalytic hydrogenation [11]. 9.6 g of diphenyl[$^2H_{25}$]dodecylphosphoric acid were dissolved in 200 ml of freshly distilled dioxane and shaken under H_2 with 0.6 g of 10% Pt on charcoal at room temperature for 36 h. The catalyst was removed by filtration and washed with warm $CHCl_3$ and CH_3OH. The dioxane filtrate and the washings were dried. NMR indicated that the product was virtually pure [$^2H_{25}$]dodecylphosphoric acid. Yield 6.0 g = 96%.

[$^2H_{13}$]Choline acetate. 5 g (74 mmol) of $N(C^2H_3)_3$ (Merck, Sharp and Dohme) was transferred under vacuum to a flask containing 150 ml dry acetone cooled on a solid CO_2/isopropanol bath. 5 g (39 mmol) of $HOC^2H_2C^2H_2Br$ (Merck, Sharp and Dohme) was dissolved in 50 ml cold, dry acetone and added dropwise. The closed vessel was allowed to warm to room temperature and stirred in the dark for two days. The acetone-insoluble material was then removed by filtration and dried under vacuum. This material was dissolved in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4) and washed through a column of Amberlite IRA-93 (Serva) in the acetate form. After drying the eluate under vacuum over P_2O_5, NMR revealed the material to be virtually pure choline acetate. Yield 6.0 g = 88%.

[$^2H_{38}$]Dodecylphosphocholine. [$^2H_{13}$]Choline acetate and [$^2H_{25}$]dodecylphosphoric acid were coupled using triisopropylbenzenesulfonyl chloride according to Aneja and Chadha [12]. 1.16 g (4 mmol) of [$^2H_{25}$]dodecylphosphoric acid and 1.67 g (10 mmol) of [$^2H_{13}$]choline acetate were mixed and dried overnight under vacuum over P_2O_5. 100 ml of dry pyridine was added followed by 3.6 g (12 mmol) of triisopropylbenzenesulfonyl chloride (Fluka).

The mixture was stirred at 70°C for 2 h and then at room temperature for 6 h. After cooling on an ice bath, unreacted choline acetate was filtered off. 5 ml of H_2O were added to the filtrate to destroy excess triisopropylbenzenesulfonyl chloride and then the solvent removed by rotary evaporation. The residual solid was extracted with $CHCl_3$ and the $CHCl_3$ extract taken to dryness. The solid material obtained was taken up in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4) and poured through a mixed-bed ion-exchange resin containing Amberlites IRC-50 and IRA-93. The eluate was dried under vacuum and then chromatographed on a column of silica gel 60 using $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4) for elution. No impurities were detected in the final product by thin-layer chromatography. Yield 0.9 g = 60%.

NMR spectroscopy. 360 MHz Fourier transform ^1H NMR spectra were recorded on a Bruker HX-360 instrument. Chemical shifts are given relative to internal sodium 3-trimethyl[2,2,3,3-^2H]propionate at pH 8 [13]. The resolution of the ^1H NMR spectra was improved by multiplication of the free induction decay with a sine bell [14]. Difference spin decoupling [15] and difference nuclear Overhauser effect [16] experiments were performed as previously described. Values of p^2H are pH meter readings uncorrected for isotope effects.

Results

^1H NMR spectra of the non-labile protons of micelle-bound melittin

Fig. 1 shows 360 MHz ^1H NMR spectra of free, monomeric melittin and of melittin bound to micelles of either deuterated dodecylphosphocholine or deuterated dodecyl-(oxyethylene)$_8$-glycol. In these spectra the melittin has been pre-exchanged with ^2H_2O and the detergents contain only approx. 1% protons so that virtually all of the observed resonances arise from non-labile protons of melittin. Comparison of the spectra in Fig. 1 shows that the resolution of the ^1H NMR resonances is comparable for free and micelle-bound melittin. This suggests that conventional high resolution NMR techniques for the study of polypeptide or protein conformation in homogeneous solution should be applicable to study of the conformation of micelle-bound melittin. To demonstrate that this expectation can be realized, NMR experiments typically used to characterize polypeptide or protein conformation in homogeneous solution are shown in the following to be practicable for micelle-bound melittin.

Initial information about the conformational state of micelle-bound melittin is provided by the spectra in Fig. 1. The major differences observed for the ^1H NMR spectra of free and micelle-bound melittin indicate that appreciable conformational changes occur upon binding melittin to micelles (see [17] for a more detailed comparison of spectral features). Since we have found that the ^1H NMR spectrum of free, monomeric melittin corresponds to a largely flexible, extended conformation (unpublished results), the differences in the spectra in Fig. 1 suggest that micelle-bound melittin has a very different conformation. Comparison of the ^1H NMR spectra of melittin bound to dodecylphosphocholine micelles or to dodecyl-(oxyethylene)$_8$-glycol micelles shows that these spectra are similar but not identical (Fig. 1, [17]). This suggests that while the overall conformation of micelle-bound melittin is determined by the location

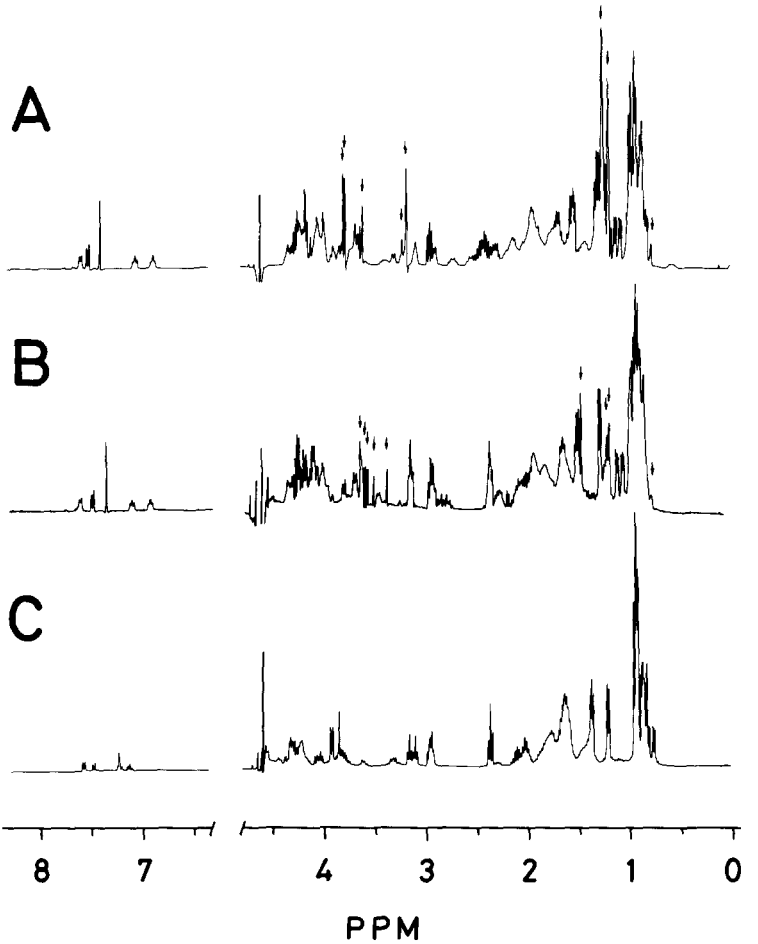

Fig. 1. 360 MHz Fourier transform ^1H NMR spectra at p^2H 5.5 and 37°C of free, monomeric melittin and of melittin bound to deuterated detergent micelles. (A) 2 mM melittin plus 90 mM [$^2H_{38}$]dodecylphosphocholine. (B) 2 mM melittin plus 120 mM [$^2H_{62}$]dodecyl-(oxyethylene)$_8$-glycol. (C) 2 mM melittin. In spectra (A) and (B) those resonances which arise from residual protons in the deuterated detergents have been marked with an arrow.

of melittin at a lipid-water interface, the properties of the interface may result in some conformational differences. Further evidence for this interpretation is described in the following.

^1H NMR spectra of the labile protons of micelle-bound melittin

In analyzing protein or polypeptide conformation in homogeneous solution by high resolution ^1H NMR, useful information is often obtained from the resonances arising from labile protons, i.e. those protons which can exchange with the solvent [18]. The spectra shown in Fig. 2 indicate that such protons can also be observed for micelle-bound melittin. Fig. 2A shows the 6—11 ppm region of the ^1H NMR spectrum of melittin bound to fully deuterated dodecylphosphocholine micelles in H_2O at pH 3.0 and 37°C. In addition to the resonances from the five non-labile protons of the indole ring of tryptophan 19

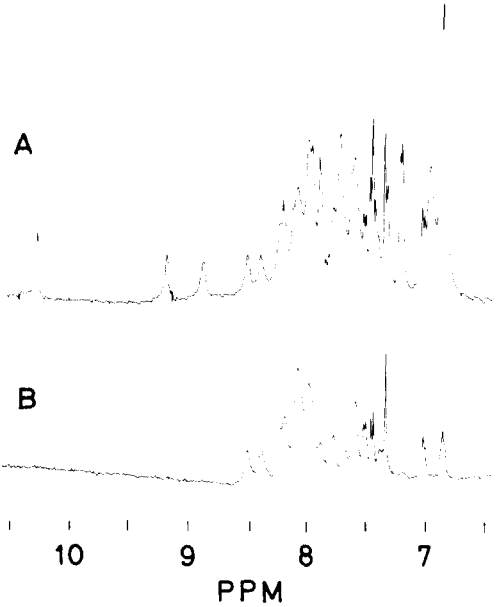

Fig. 2. 360 MHz Fourier transform ^1H NMR spectra of melittin bound to deuterated dodecylphosphocholine micelles showing the resonances of the aromatic and labile protons of melittin. (A) 4 mM melittin plus 180 mM [$^2H_{38}$]dodecylphosphocholine in H_2O at pH 3.0 and 37°C. (B) 4 mM melittin plus 180 mM [$^2H_{38}$]dodecylphosphocholine 20 min after dissolving in ^2H_2O at p^{2}H 2.8 and 37°C.

(see Fig. 1), a variety of resonances from backbone amide NH protons and from the labile protons of amino acid side chains are observed. Melittin has a total of 54 labile protons, including 25 backbone amide NH protons, which could give resonances between 6 and 11 ppm. The total intensity in this spectral range in Fig. 2A corresponds to approx. 40 protons, i.e. approx. 35 labile protons, which indicates that a significant proportion of the labile protons of melittin contribute to the observed spectrum. Some of the labile protons are apparently not observable because of rapid exchange on the NMR time scale with the solvent H_2O, indicating that even in the micelle-bound form some of the labile protons of melittin must be freely accessible to the solvent.

In random-coil polypeptides, the resonances of the labile protons of the amino acid side chains present in melittin have chemical shifts between 6.6 and 7.6 ppm in H_2O [19]. The only exceptions to this are the hydroxyl protons of serine and threonine, which are not normally observed because of rapid exchange with H_2O, and the NH proton of the indole ring of tryptophan which gives a resonance at 10.2 ppm in random coil tetrapeptides [19] and which is observed at 10.3 ppm for micelle-bound melittin (Fig. 2A). These considerations indicate that many of the resonances observed between 7.6 and 9.3 ppm for micelle-bound melittin arise from backbone amide NH protons. Since in random-coil tetrapeptides [19] and in free, monomeric melittin (unpublished results), the backbone amide NH protons of the amino acids contained in melittin have resonance positions between 8.1 and 8.5 ppm, it appears that a number of these protons have somewhat different chemical shifts in micelle-bound melittin (Fig. 2A).

When protonated melittin was bound to dodecylphosphocholine micelles in H_2O, lyophilized and redissolved in ^2H_2O at p^{2}H 2.8 and 37°C, the spectrum shown in Fig. 2B was observed 20 min after dissolution of the melittin-dodecylphosphocholine complex in ^2H_2O. Comparison with Fig. 2A shows that the resonance at 10.3 ppm from the NH proton of the indole ring of tryptophan 19 and virtually all resonances between 6.6 and 7.6 ppm, excepting the resonances from the non-labile protons of tryptophan 19, are no longer observable. The labile protons that gave rise to these resonances in H_2O have exchanged with ^2H_2O in less than 20 min, which indicates that for micelle-bound melittin the labile protons of the side chains of lysine, arginine, glutamine and tryptophan must be accessible to the solvent. In contrast, for the backbone amide NH resonances between 7.6 and 9.3 ppm in Fig. 2A, an intensity corresponding to approx. 15 labile protons is observed after 20 min in ^2H_2O at p^{2}H 2.8 and 37°C (Fig. 2B). Most of these backbone amide NH protons of micelle-bound melittin were observed to completely exchange with solvent deuterons after approx. 10—15 h, however, the two one-proton resonances at 8.4 and 8.6 ppm disappeared completely only after several days. Since for random-coil polypeptides [19,20] and for free, monomeric melittin (unpublished results), all labile protons exchange with solvent ^2H_2O within several minutes under these conditions, these results indicate that an appreciable proportion of the backbone amide NH protons of micelle-bound melittin are not readily accessible to the solvent.

Resonance assignments and nuclear Overhauser effects in micelle-bound melittin

A pre-requisite for conformational analysis of polypeptides or proteins by high resolution NMR is the assignment of the resonances in the NMR spectrum to specific nuclei within the polypeptide or protein. The first level of assignment, which is normally based on spectroscopic features, is the assignment of resonances to particular types of amino acids. This level of assignment has been achieved for a considerable number of the ^{1}H NMR resonances of micelle-bound melittin. These assignments will be presented in detail elsewhere, but an example, which shows that conventional high-resolution NMR techniques are feasible for micelle-bound melittin and which gives some information about conformational properties of micelle-bound melittin, is presented here.

Fig. 3 shows spectra from NMR experiments on melittin bound to fully deuterated dodecylphosphocholine micelles which have been used to identify complete spin systems, exclusive of labile protons, of the two valine residues at positions 5 and 8 of the amino acid sequence. Fig. 3B shows that decoupling at 2.33 ppm, the region of the ^{1}H NMR spectrum expected to contain the resonances corresponding to the βCH protons of valine, results in simultaneous decoupling of four methyl doublet resonances at 1.160, 1.108, 1.004 and 0.980 ppm and of two one-proton doublets at 3.824 and 3.693 ppm. This result suggests that the βCH protons of both valine 5 and valine 8 give resonances very close to 2.33 ppm and that the αCH doublet and two γCH_3 doublets of both valine residues have been decoupled. This has been confirmed, and the αCH and γCH_3 resonances correlated with one another, by the use of nuclear Overhauser effect difference spectra [16]. Pre-saturation of either the

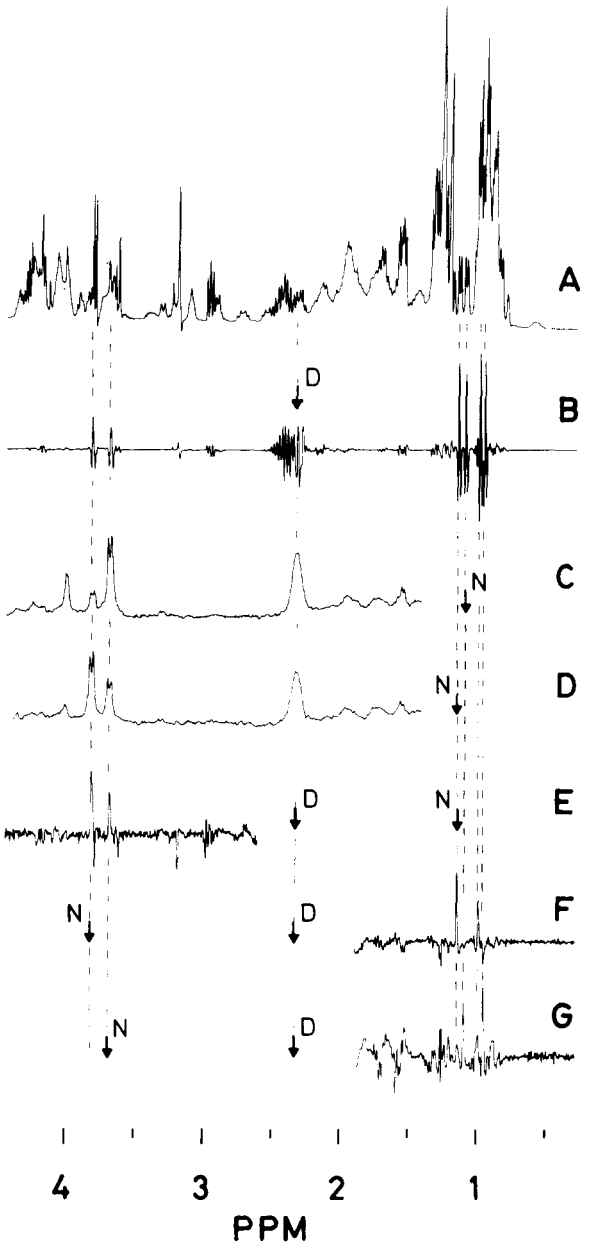

Fig. 3. 360 MHz Fourier transform ^{1}H NMR spectra of melittin bound to deuterated dodecylphosphocholine micelles showing difference double-resonance experiments used to assign the spin systems of valine residues 5 and 8 of melittin. The arrows show the frequencies where double-resonance irradiation was applied. N, weak irradiation applied for 0.4—0.6 s prior to data acquisition in order to build up nuclear Overhauser effects. D, decoupling irradiation applied in time-sharing mode during data acquisition. (A) The high-field region of the ^{1}H NMR spectrum of 8 mM melittin plus 360 mM [$^2H_{38}$]dodecylphosphocholine at pH 5.5 and 37°C. (B) The difference between spectra with or without decoupling of the valine βCH resonances at 2.33 ppm. (C) The difference between spectra with or without build up of nuclear Overhauser effects by irradiation of the valine γCH_3 doublet at 1.108 ppm. (D) The difference between spectra with or without build up of nuclear Overhauser effects by irradiation of the valine γCH_3 doublet at 1.160 ppm. (E) The difference between spectra with decoupling at the valine βCH resonances at 2.33 ppm and with or without build up of nuclear Overhauser effects by irradiation of the valine γCH_3 doublet at 1.160 ppm. (F) The difference between spectra with decoupling at the valine βCH resonances

methyl doublet resonance at 1.108 ppm (Fig. 3C) or the methyl doublet resonance at 1.160 ppm (Fig. 3D) results in nuclear Overhauser effects at the βCH resonance at 2.33 ppm and at both αCH resonances at 3.824 and 3.693 ppm. Repetition of the experiment in Fig. 3D with the addition of decoupling at the position of the βCH resonances resulted in decoupling of both αCH resonances (Fig. 3E), thereby confirming that the same αCH resonances are observed in the difference decoupling (Fig. 3B) and difference nuclear Overhauser effect (Fig. 3C—E) experiments. Since the frequency difference between the two methyl groups at 1.108 and 1.160 ppm is small, the pre-saturation of these resonances was not completely selective, however, the magnitude of the nuclear Overhauser effect observed for the αCH resonances in the two experiments (Fig. 3C and D) indicated that the αCH resonance at 3.693 ppm and the γCH_3 resonance at 1.108 ppm correspond to one valine and the αCH resonance at 3.824 ppm and the γCH_3 resonance at 1.160 ppm correspond to the other valine. This has been confirmed, and the remaining two methyl groups correlated with the αCH resonances, by pre-saturation of the αCH resonances combined with decoupling of the βCH resonances. Pre-saturation at 3.824 ppm and decoupling at 2.33 ppm gave two methyl singlet resonances at 1.160 and 1.004 ppm (Fig. 3F). Pre-saturation of the other valine αCH at 3.693 ppm, which lies in a very crowded region of the spectrum, and decoupling at 2.33 ppm gave a number of resonances in the methyl region, however, two singlet resonances at 1.108 and 0.980 ppm were clearly observable (Fig. 3G). The chemical shifts, spin-spin coupling constants and calculated rotamer populations for the two valine residues of micelle-bound melittin and for valine in random-coil tetrapeptides [19] are shown in Table I. Analysis of these data are deferred to Discussion.

A further interesting aspect of these experiments was the magnitude and selectivity of the observed nuclear Overhauser effects. As can be seen in Fig. 3C and D, with irradiation of the valine γCH_3 resonances for short times (0.4—0.6 s), most of the observed nuclear Overhauser effect was restricted to other protons of the valine side chains. Under these conditions the nuclear Overhauser effects at the αCH and βCH protons of valine were small and negative ($I/I_0 \approx 0.90$—0.95). For irradiation of the valine γCH_3 resonances for much longer periods (4—6 s), the intensity changes at the αCH and βCH resonances were somewhat larger ($I/I_0 \approx 0.85$—0.90). For long periods of pre-saturation, intensity changes were also observed at some other resonances although the valine resonances continued to account for a major proportion (approx. 30—35%) of the total nuclear Overhauser effect observed. Similar results were obtained for pre-saturation of the βCH resonances. For short irradiation periods (0.6 s), small, negative nuclear Overhauser effects were observed for the αCH and γCH_3 resonances ($I/I_0 \approx 0.90$). For long periods of irradiation (4—6 s), the

at 2.33 ppm and with or without build up of nuclear Overhauser effects by irradiation of the valine αCH doublet at 3.824 ppm. (G) The difference between spectra with decoupling at the valine βCH resonances at 2.33 ppm and with or without build up of nuclear Overhauser effects by irradiation of the valine αCH doublet at 3.693 ppm. All observed nuclear Overhauser effects were negative, but have been plotted as positive differences to facilitate comparison. Because the nuclear Overhauser effects were small (see text), the large resonance intensity at the frequency were the nuclear Overhauser effect was built up and the disturbances near the decoupling frequency have been omitted from plots C to G.

TABLE I

^{1}H NMR PARAMETERS FOR VALINE IN MELITTIN BOUND TO DODECYLPHOSPHOCHOLINE MICELLES AND IN RANDOM-COIL TETRAPEPTIDES

Population of *gauche* and *trans* rotamers about the χ_1 dihedral angle calculated from $J_{\alpha\beta}$ and the Karplus-type equation of 21.

Residue	Chemical shift (ppm)			Coupling constant (Hz)		Rotamer population	
	αCH	βCH	γCH_3	$^3J_{\alpha\beta}$	$^3J_{\beta\gamma}$	P_{gauche}	P_{trans}
Micelle-bound melittin							
Val 5 or 8	3.824	2.33	1.160	8.8	6.4	0.43	0.57
			1.004		6.4		
Val 5 or 8	3.693	2.33	1.108	9.8	6.4	0.33	0.67
			0.980		6.4		
Random-coil tetrapeptide *							
Val 3	4.184	2.13	0.969	6.9	6.9	0.63	0.37
			0.942		6.9		

* Ref. 19.

intensity changes at the αCH and γCH_3 resonances were larger ($I/I_0 \approx 0.85$). Again, intensity changes at some other resonances were observed, but the valine αCH and γCH_3 resonances accounted for approximately 50% of the total nuclear Overhauser effect observed. In contrast, when the βCH resonances of valine were irradiated in free, monomeric melittin, little or no intensity change was observed at the αCH protons and small, positive intensity changes were observed for the γCH_3 resonances ($I/I_0 \approx 1.03$). As will be seen in Discussion, these nuclear Overhauser effects allow some initial conclusions about the motional characteristics of the valine side chains in micelle-bound melittin.

Temperature dependence of the ^{1}H NMR spectrum of micelle-bound melittin

In homogeneous solution the high-information content of the NMR method has often been used to monitor the location and nature of conformational changes undergone by proteins or polypeptides [18]. An example of this type of experiment for micelle-bound melittin is shown in Fig. 4 where the observed chemical shifts as a function of temperature are plotted for a variety of resonances from melittin bound to fully deuterated dodecylphosphocholine micelles. In Fig. 4, the methyl resonances from alanines 4 and 15, threonines 10 and 11 and valines 5 and 8 are shown to have constant chemical shifts over the temperature range 5–90°C, suggesting that these residues have a constant conformational state over this entire temperature range. The ϵCH_2 resonances of the lysine residues 7, 21 and 23, the δCH_2 resonance of arginine 22 or 24 and the H2, H6 and H5 resonances of the indole ring of tryptophan 19 show continuous changes in chemical shift between 5 and 90°C suggesting these residues are in rapid equilibrium on the NMR time scale between at least two different conformational states. Below approx. 40°C, the other arginine δCH_2 resonance broadens and decreases in intensity while the γCH_2 protons of both glutamines 25 and 26 become inequivalent suggesting that below 40°C these residues are in slow exchange on the NMR time scale between at least two different conformational states. In Discussion these results are related to the

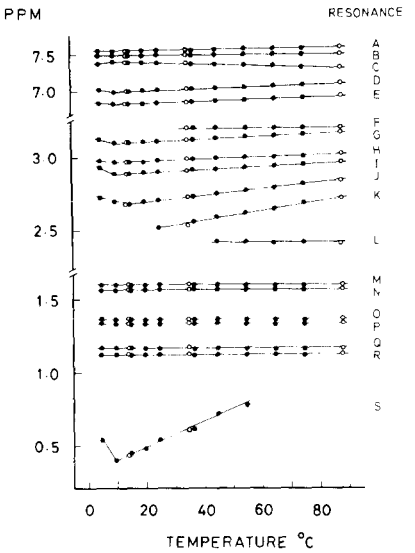

Fig. 4. Chemical shifts for the resonances of melittin bound to deuterated dodecylphosphocholine micelles at pH 5.5 as a function of temperature. •, 2 mM melittin plus 90 mM [$^2H_{38}$]dodecylphosphocholine. ○, 2 mM melittin plus 120 mM [$^2H_{38}$]dodecylphosphocholine. The assignments of the resonances are: A—E, H4, H7, H2, H6, H5 of the indole ring Trp 19; F and G, Arg 22 and 24, δCH_2; H—K, Lys 7, 21 and 23, ϵCH_2. J and K are one proton resonances; L, Gln 25 and 26, γCH_2; M and N, Ala 4 and 15, βCH_3; O and P, Thr 10 and 11, γCH_3; Q and R, Val 5 and 8, γCH_3, and S, unassigned one-proton resonance.

melittin amino acid sequence and to our previous conclusions [17] about the nature of the melittin-micelle interaction.

Discussion

The use of micellar detergent-protein complexes for ^1H NMR studies of the conformation of membrane proteins requires that: (1) the conformation of the membrane protein is preserved in a micelle; (2) the micellar complex is homogeneous and stoichiometrically well-defined, and (3) a ^1H NMR spectrum of sufficient quality to permit detailed analysis is obtained. Using a variety of physical methods, we have previously shown that melittin has a very similar conformation when bound to micelles of various zwitterionic or non-ionic detergents, to micelles of α-L-diheptanoylphosphatidylcholine and to bilayers of α-L-dilauroylphosphatidylcholine [17]. It was also shown that melittin forms homogeneous complexes with dodecylphosphocholine consisting of one melittin molecule and about forty detergent molecules [17]. It is evident from the present experiments that the ^1H NMR spectrum obtained for melittin bound to fully deuterated dodecylphosphocholine micelles is amenable to detailed analysis and therefore that multi-parameter information on the conformation of micelle-bound melittin, and implicitly on the conformation of membrane-bound melittin, is accessible.

Other membrane polypeptides and proteins can be studied in similar fashion. Well-resolved ^1H NMR spectra have been obtained for glucagon bound to

dodecylphosphocholine micelles and this complex has also been found to be stoichiometrically well-defined (Boesch, C., Brown, L.R. and Wüthrich, K., unpublished results). Evidence is available which indicates similar conformations for glucagon bound to various types of micellar or bilayer lipid aggregates [22, 23]. Preliminary experiments with porcine pancreatic phospholipase A_2, a globular protein of 124 amino acids, have shown that the ^1H NMR spectra of the free protein and of the protein bound to deuterated dodecylphosphocholine micelles show comparable resolution (Chrezeszczyk, A., Brown, L.R., de Haas, G.H. and Wüthrich, K., unpublished results). Previous work has shown that pancreatic phospholipases A_2 bind to hexadecylphosphocholine and to substrate analogues, either as monomers or micelles, in similar fashion [24] and that homogeneous complexes are obtainable with hexadecylphosphocholine micelles [25]. Thus, the micellar complexes formed between fully deuterated detergents and melittin, glucagon or porcine pancreatic phospholipase A_2 all appear to satisfy the above requirements for high resolution ^1H NMR studies of the conformation of membrane-bound proteins.

The above examples suggest that for many membrane-bound proteins high resolution ^1H NMR studies of the complexes formed between membrane proteins and fully deuterated micelles provide a viable method for obtaining multiparameter conformational data which should be useful for interpretation of the functional properties of membrane-bound proteins. To illustrate the potential of this approach, in the following evidence is presented that interaction with micelles results in different conformational and dynamic features for different regions of the polypeptide chain of bound melittin.

The amino acid sequence of melittin shows a pronounced amphiphilic distribution of polar and non-polar amino acids [26].

Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-
1 5 10 16 20

Arg-Gln-Gln-NH_2
25

Thus, residues 1—20 are predominantly non-polar whereas residues 21—26 are polar and carry four positive charges. In a previous study, comparison of NMR data for melittin bound to various types of protonated detergent or phospholipid micelles suggested that the C-terminal region of micelle-bound melittin was largely exterior to the micelle, the N-terminal region penetrated into the micelle interior and the indole ring of tryptophan 19 lay in the interfacial region [17]. The present experiments provide results which support these conclusions.

The rapid exchange with solvent ^2H_2O observed for the labile protons of the side chains of the lysine, arginine, glutamine and tryptophan residues (Fig. 2) indicates that the side chains of the C-terminal residues of melittin are accessible to the solvent. Furthermore, for melittin bound to dodecylphosphocholine micelles the temperature dependence of the ^1H NMR spectrum indicates that these C-terminal residues have considerable conformational variability whereas the alanine, threonine and valine residues located in the N-terminal hydrophobic region of melittin appear to have a single conformational state between 5 and 90°C (Fig. 4). In our previous study [17], comparison of NMR and

circular dichroism measurements suggested that a major portion of the N-terminal region of micelle-bound melittin assumes an α-helical conformation. Observation of numerous backbone amide NH resonances which exchange slowly with solvent ^2H_2O (Fig. 2) and which have chemical shifts appreciably different from the chemical shifts observed for random-coil polypeptides [19] would be consistent with this suggestion. Definitive proof of this suggestion will require assignment of the backbone amide NH resonances in the ^1H NMR spectrum of micelle-bound melittin.

Further information on the conformational state of the hydrophobic region of micelle-bound melittin is provided by the experiments used to identify the spin systems of valines 5 and 8. The considerable differences in the NMR parameters observed for the valine residues of micelle-bound melittin compared to random-coil tetrapeptides (Table I) indicates that the valine side chains of micelle-bound melittin have a non-random conformation. The $^3J_{\alpha\beta}$ coupling constants for the side chains of valines 5 and 8 (Table I) can be interpreted either as indicating fixed χ_1 dihedral angles of approx. 0° or 140° for each valine or as indicating rapid equilibrium on the NMR time scale between *gauche* and *trans* rotamers with the population distribution shown in Table I. If the observed coupling constants correspond to the rapid equilibrium case, the rate of interconversion of the *gauche* and *trans* rotamers must be at least approx. $10^3\ s^{-1}$ and may be much faster. For the valine side chains, observation of positive nuclear Overhauser effects for free, monomeric melittin and negative nuclear Overhauser effects for micelle-bound melittin indicates reduced rotational motion upon binding melittin to the micelle [27]. On the other hand, from our previous hydrodynamic characterization of the melittin-dodecylphosphocholine complex [17], a rotational correlation time of approx. $1 \cdot 10^{-8}$ s can be calculated. Since the magnitudes of the observed nuclear Overhauser effects were small and rather selective, these results suggest that the rotational rates of the valine side chains are faster than that of the micelle as a whole [27]. Further experiments to verify this suggestion and to characterize spin diffusion in micelle-bound melittin are in progress, however, this is not a surprising result since fast rotational motions are observed for aliphatic side chains in the interior of globular proteins [28,29].

The results of this study and of a previous study [17] suggest the following qualitative picture of the melittin-micelle interaction. The hydrophilic C-terminal sequence of melittin lies at the micelle surface and has conformational mobility probably involving both side chain and backbone torsion angles. The hydrophobic N-terminal sequence of melittin penetrates into the micelle interior and appears to have a definite backbone conformation but considerable rotational freedom of side chains. The nature of the lipid-water interface has little influence on the conformational state of the N-terminal region, but causes some change in the conformational state of the C-terminal region. Further experiments to define the conformation of micelle-bound melittin which leads to these qualitative features are in progess.

Acknowledgements

I thank Dr. M. Chabre for a sample of [$^2H_{62}$]dodecyl-(oxyethylene)$_8$-glycol and Prof. K. Wüthrich for continued interest and support. Financial support by

the Swiss National Science Foundation (project 3.004.0.76) is gratefully acknowledged.

References

1 Brown, L.R., Bradbury, J.H., Austin, K. and Stewart, P.R. (1975) J. Membrane Biol. 24, 35—54
2 Austin, K., Brown, L.R. and Stewart, P.R. (1975) J. Membrane Biol. 24, 55—69
3 Brown, L.R. and Wüthrich, K. (1977) Biochim. Biophys. Acta 468, 389—410
4 Dufourcq, J., Bernon, R. and Lussan, C. (1976) Biochim. Biophys. Acta 433, 252—263
5 Feigenson, G.W., Meers, P.R. and Kingsley, P.B. (1977) Biochim. Biophys. Acta 471, 487—491
6 King, T.P., Sobotka, A.K., Kochoumian, L. and Lichtenstein, L.M. (1976) Arch. Biochem. Biophys. 172, 661—671
7 Mollay, C., Kreil, G. and Berger, H. (1976) Biochim. Biophys. Acta 426, 317—324
8 Dinh-Nguyen, N., Raal, A. and Stenhagen, E. (1972) Chem. Scr. 2, 171—178
9 Anhoury, M.L., Arickx, M., Crooy, P., De Neys, R. and Eliaers, J. (1974) J. Chem. Soc., Perkin I, 191—192
10 Baer, E. (1951) J. Biol. Chem. 189, 235—247
11 Van der Nuet, J.H., Uhlenbroek, J.H. and Verkade, P.E. (1953) Rec. Trav. Chim. 72, 365—376
12 Aneja, R. and Chadha, J.S. (1971) Biochim. Biophys. Acta 248, 455—457
13 De Marco, A. (1977) J. Magn. Resonance 26, 527—528
14 De Marco, A. and Wüthrich, K. (1976) J. Magn. Resonance 24, 201—204
15 De Marco, A., Tschesche, H., Wagner, G. and Wüthrich, K. (1977) Biophys. Struct. Mech. 3, 303—315
16 Richarz, R. and Wüthrich, K. (1978) J. Magn. Resonance 30, 147—150
17 Lauterwein, J., Boesch, Ch., Brown, L.R. and Wüthrich, K. (1979) Biochim. Biophys. Acta, in the press
18 Wüthrich, K. (1976) NMR in Biological Research: Peptides and Proteins, North-Holland, Amsterdam
19 Bundi, A. and Wüthrich, K. (1979) Biopolymers 18, 285—297
20 Englander, S.W., Downer, N.W. and Teitelbaum, H. (1972) Annu. Rev. Biochem. 41, 903—924
21 De Marco, A., Llinás, M. and Wüthrich, K. (1978) Biopolymers 17, 617—636
22 Schneider, A.B. and Edelhoch, H. (1972) J. Biol. Chem. 247, 4992—4995
23 Epand, R.M., Jones, A.J.S. and Schreier, S. (1977) Biochim. Biophys. Acta 491, 296—304
24 Van Dam-Mieras, M.C.E., Slotboom, A.J., Pieterson, W.A. and de Haas, G.H. (1975) Biochemistry 14, 5387—5394
25 Soares de Araujo, P., Rosseneu, M.Y., Kremer, J.M.H., van Zoelen, E.J.J. and de Haas, G.H. (1979) Biochemistry 18, 580—586
26 Habermann, E. and Jentsch, J. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 37—50
27 Bothner-By, A.A. and Johner, P.E. (1978) Biophys. J. 24, 779—790
28 Nelson, D.J., Opella, S.J. and Jardetzky, O. (1976) Biochemistry 25, 5552—5560
29 Nigen, A.M., Keim, P., Marshall, R.C., Glushko, V., Lawson, P.J. and Gurd, F.R.N. (1973) J. Biol. Chem. 248, 3716—3723