

Supporting Information

Elucidation of the Structure of the Membrane Anchor of Penicillin-Binding Protein 5 of *Escherichia coli*

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CH Sepharose 4B resin, Triton X-100, ampicillin, kanamycin, sodium dodecylsulfate (SDS) and [3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) were purchased from Sigma. All restriction enzymes and other DNA-modifying enzymes were either from New England Biolabs or Stratagene. Isopropyl- β -D-thiogalactoside (IPTG) were purchased from Fisher Scientific. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), 1,1',2,2'-tetraoleoyl cardiolipin (CL) and dodecylphosphocholine (DPC) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Phospholipid concentrations were measured by phosphate analysis (29). The Liposofast microextruder and 100-nm polycarbonate filters were purchased from Avestin (Ottawa, Ontario). Urea and octyl glucoside were from Fisher Scientific. Pioneer L1 sensor chip was purchased from Biacore AB (Piscataway, NJ).

Table S1. NMR and refinement statistics for protein structures.

	PBP 5 Anchor
NMR distance and dihedral constraints	
Distance constraints	
Total NOE	422
Intra-residue	117
Inter-residue	305
Sequential ($ i - j = 1$)	238
Medium-range ($ i - j < 4$)	168
Long-range ($ i - j > 5$)	16
Intermolecular	
Hydrogen bonds	10
Total dihedral angle restraints	38
ϕ	19
ψ	19
Structure statistics	
Violations (mean and s.d.)	
Distance constraints (Å)	0.176 ± 0.097
Dihedral angle constraints (°)	10.30 ± 3.62
Max. dihedral angle violation (°)	10.36 ± 3.62
Max. distance constraint violation (Å)	0.178 ± 0.097
Average pairwise r.m.s. deviation** (Å)	
Heavy	0.52 ± 0.11
Backbone	0.32 ± 0.17

Table S2. ¹H NMR chemical shifts δ for the PBP 5 anchor peptide in an aqueous buffered solution pH 7.4 in the presence of DPC micelles.

Residue	NH	H α	H β	H γ or H2,6	H δ or H3,5	H ϵ or H4
Glu-1	8.384	4.186	1.995,1.877	2.226,2.219		
Gly-2	8.554	3.891				
Asn-3	8.32	4.746	2.896,2.817	7.037,7.672		
Phe-4	8.755	4.187	3.207,2.837	6.953	7.124	7.038
Phe-5	8.601	4.107	3.138,3.043	7.245	7.14	6.953
Gly-6	8.072	3.865,3.642				
Lys-7	7.705	4.088	1.838,1.694	1.494,1.387	1.663,1.583	2.878
Ile-8	7.59	3.63	1.948	0.824/1.476,0.878	0.643	
Ile-9	7.951	3.521	1.928	0.852/1.448,1.021	0.666	
Asp-10	8.105	4.311	2.72,2.501			
Tyr-11	7.939	4.219	3.138/3.035	6.952	6.68	
Ile-12	8.407	3.581	2.035	0.856/1.111	0.796	
Lys-13	8.503	3.814	1.934,1.853	1.202,1.709	1.705	2.821,2.770
Leu-14	7.838	4.079	1.715	1.563	0.819/0.782	
Met-15	8.157	4.053	1.949	2.247,2.086		
Phe-16	8.661	4.295	3.214,3.137	6.953	7.05	7.14
His-17	8.362	4.238	3.242	6.963 (4)	7.954 (2)	
His-18	8.232	4.219	3.019	6.362 (4)	7.789 (2)	
Trp-19	8.153	4.225	2.871,	6.842 10.471	7.362 7.00	7.234 6.874
Phe-20	8.161	4.438	3.026,2.394	7.137	7.062	7.01
Gly-21	7.689	3.820,3.762				
NH ₂	7.157, 7.090					

Table S3. ^{15}N and ^{13}C NMR chemical shifts δ for the PBP 5 anchor peptide in an aqueous buffered solution pH 7.4 in the presence of DPC micelles.

Residue	N	C α	C β	C γ or C2,6	C δ or C3,5	C ϵ or C4
Glu-1	126.422	54.19	27.68	33.58		
Gly-2	109.81	42.832				
Asn-3	118.735	50.401	36.317	112.785		
Phe-4	122.708	58.638	36.921	129.211	128.231	126.44
Phe-5	117.326	58.467	35.874	128.335	126.547	129.551
Gly-6	106.074	44.675				
Lys-7	120.36	56.159	29.44	22.48	26.57	39.317
Ile-8	119.297	62.385		14.811/26.049	10.82	
Ile-9	119.527	62.138		15.10/26.17	9.34	
Asp-10	119.391	55.106	37.54			
Tyr-11	120.959	59.071	35.86	129.798	115.32	
Ile-12	119.653	62.939	34.763	15.063/30.96	10.94	
Lys-13	118.827	58.47	29.8	23.655	27.285	39.136
Leu-14	120.142	55.54	39.31	24.08	21.915/21.957	
Met-15	118.735	54.949		29.45		
Phe-16	119.251	59.265	36.491	129.213	128.894	128.123
His-17	118.24	57.138	27.167	117.435	135.283	
His-18	118.738	56.563	27.67	116.445	135.788	
Trp-19	117.611	59.190	27.12	122.547 129.424	111.74 121.01	117.71 118.38
Phe-20	114.139	55.576	37.141	128.118	128.901	126.812
Gly-21	108.505	42.624				
NH2	106.546					

Table S4. PBP 5 anchor hydrogen bonding vs. hydrogen/deuterium exchange.

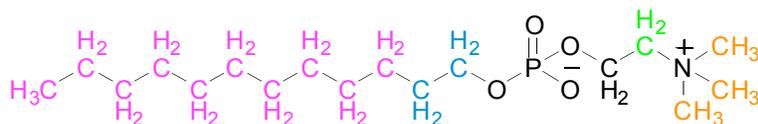
Hydrogen donor	Hydrogen acceptor	Distance Å	$t_{1/2}$ Exchange time
Gly6 NH	Asn3 CO	2.3	< 15 min
Lys7 NH	Asn3 CO	2.5	< 15 min
Ile8 NH	Phe4 CO	2.5	< 15 min
Ile9 NH	Gly6 CO	2.2	38 min
Lys13 NH	Asp10 CO	1.9	15 min
Met15 NH	Tyr11 CO	1.8	15 min
Phe16 NH	Lys13 CO	2.7	204 min
His17 NH	Leu14 CO	2.8	< 15 min
Trp19 NH	Met15 CO	2.1	< 15 min
Trp19 NH	Phe16 CO	2.5	< 15 min
Phe20 NH	Phe16 CO	1.9	< 15 min
Gly21 NH	Phe16 CO	2.1	< 15 min
His18 imidazole H	Met15 CO	2.3	-
Tyr11 NH	Asp10 γ CO ₂	1.9	-
Asp10 NH	^a		15 min
Leu14 NH	^b		15 min
Ile12 NH	^c		63.5 h

^a Asp10 H_N is in the bend region of the peptide and the backbone is twisted and the residue is 2.0 Å away from Lys7 but out of orientation for H-bonding. ^bLeu14 H_N is just before the sharp turn in the backbone and is within 2.8 and 2.0 Å of Asp10 or Tyr11 respectively but is also not orientated to properly H-bond with either residue. ^cIle12 H_N is inserted into the membrane but is oriented towards the bend with residues too far away to hydrogen bond with any residue.

Table S5. NOE interactions between the PBP 5 anchor peptide and DPC micelle protons.^a

Residue	NH	H α	H β	H γ or H2,6	H δ or H3,5	H ϵ or H4
Glu-1	8.384	4.186	1.995,1.877	2.226,2.219		
Gly-2	8.554	3.891				
Asn-3	8.32	4.746	2.896,2.817			
Phe-4	8.755	4.187	3.207,2.837	6.953	7.124	7.038
Phe-5	8.601	4.107	3.138,3.043	7.245	7.14	6.953
Gly-6	8.072	3.865,3.642				
Lys-7	7.705	4.088	1.838,1.694	1.494,1.387	1.663,1.583	2.878
Ile-8	7.59	3.63	1.948	0.824/1.476,0.878	0.643	
Ile-9	7.951	3.521	1.928	0.852/1.448,1.021	0.666	
Asp-10	8.105	4.311	2.72,2.501			
Tyr-11	7.939	4.219	3.138/3.035	6.952	6.68	
Ile-12	8.407	3.581	2.035	0.856/1.111	0.796	
Lys-13	8.503	3.814	1.934,1.853	1.202,1.709	1.705	2.821,2.770
Leu-14	7.838	4.079	1.715	1.563	0.819/0.782	
Met-15	8.157	4.053	1.949	2.247,2.086		
Phe-16	8.661	4.295	3.214,3.137	6.953	7.05	7.14
His-17	8.362	4.238	3.242	6.963(4)	7.954(2)	
His-18	8.232	4.219	3.019	6.362 (4)	7.789 (2)	
Trp-19	8.153	4.225	2.871,2.482	6.842 10.471	7.362 7.00	7.234 6.874
Phe-20	8.161	4.438	3.026,2.394	7.137	7.062	7.01
Gly-21	7.689	3.820,3.762				
NH ₂	7.038, 7.672					

^a The peptide protons with chemical shifts in black have no NOE interaction with the micelle protons or their NOE interactions overlap with the intra-peptide NOEs. Peptide protons with chemical shifts highlighted in purple exhibit NOEs with red and blue DPC protons. Peptide protons with chemical shifts highlighted in brown exhibit NOEs with green and blue DPC protons. Peptide protons with chemical shifts highlighted in grey exhibit NOEs with green and orange DPC protons. Figure S1 shows the structure of DPC with the color coding of the protons that interact with the peptide protons.

**Figure S1. DPC structure with protons color coded according to ¹H NMR NOE interactions with the peptide. DPC protons in black signify no data or NOE interaction.**

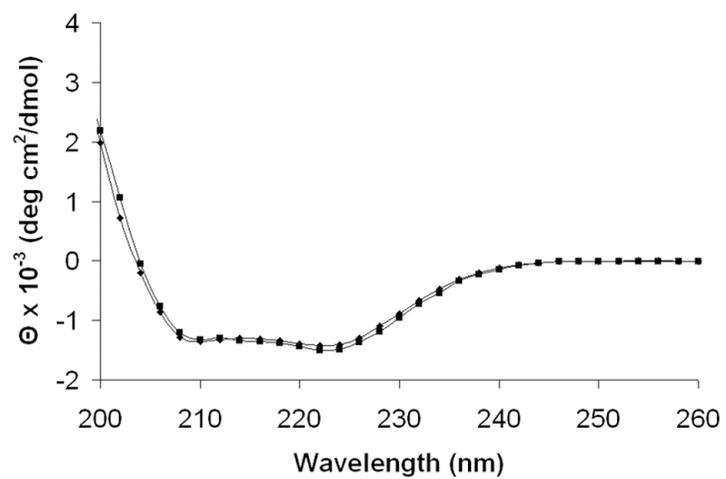


Figure S2. Comparison of the CD spectrum of the PBP 5 anchor in DPC micelles ◆ and POPE/POPG/CL lipid mixture ■ at pH 7.5.

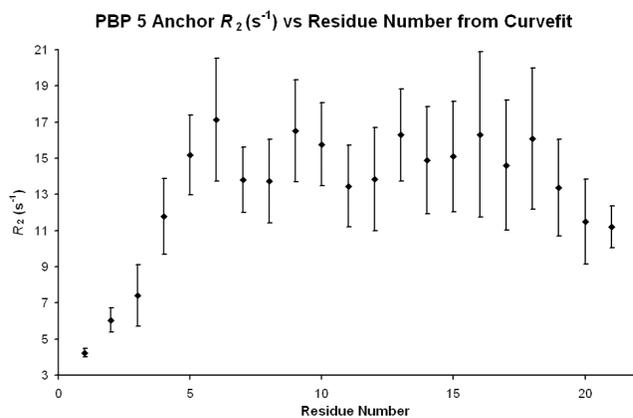
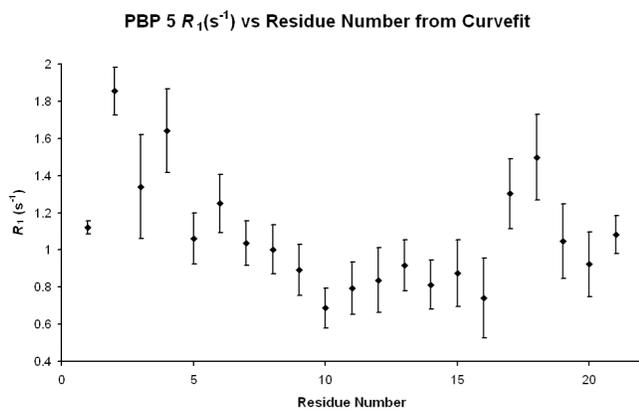


Figure S3. ^{15}N plots of R_1 and R_2 for the PBP 5 anchor of *E. coli* as a function of residue number.

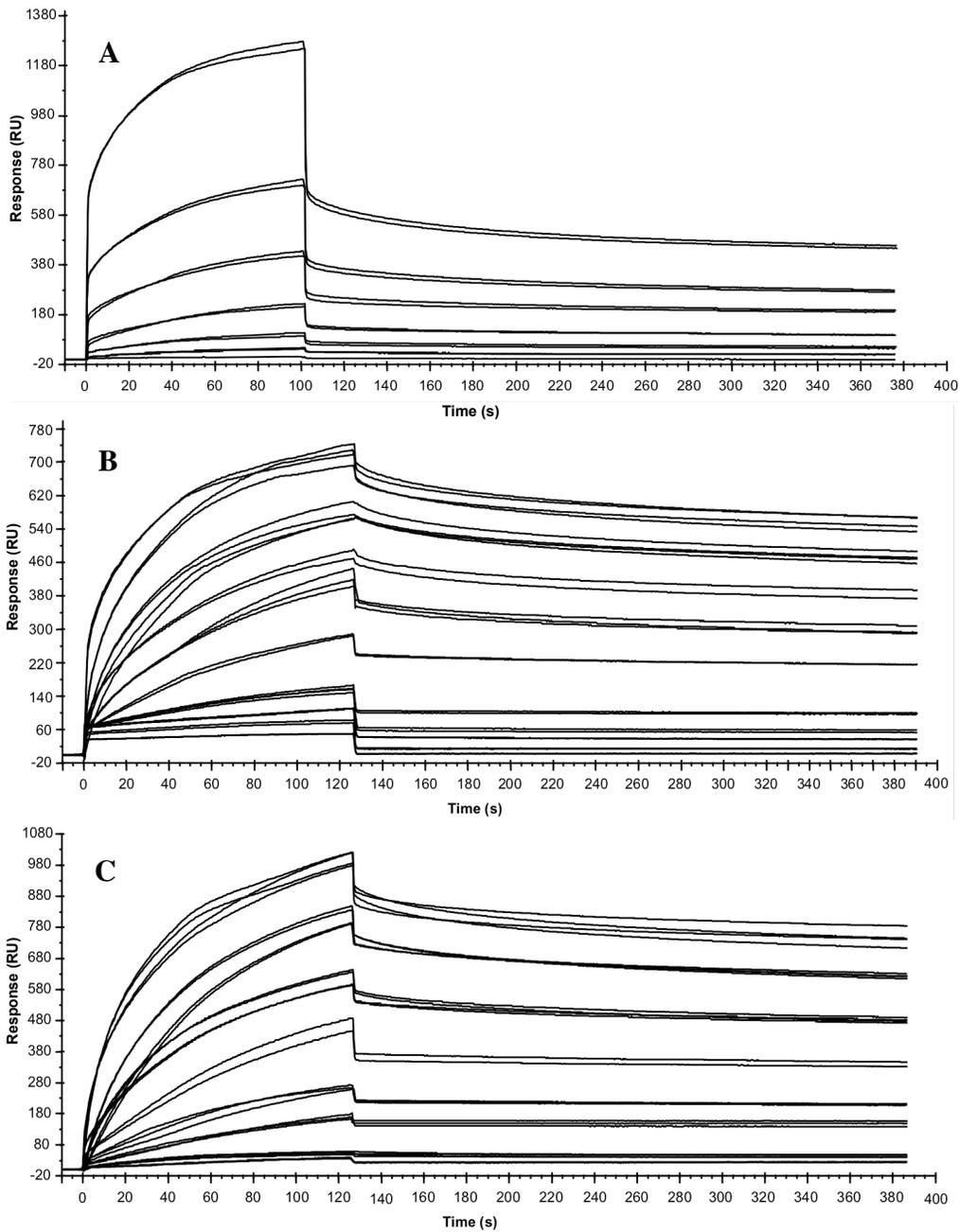


Figure S3. SPR plots of interactions of full-length PBP 5 to different lipids in HEPES buffer pH 7.4. (A) POPE:POPG:CL and Anchor PBP5 Interactions (B) DPC and Anchor PBP5 Interactions (C) SDS and Anchor PBP5 Interactions

NMR Methods

All deuterated reagents and solvents were from Cambridge Isotopes unless otherwise noted. To form a 2 mM solution, two milligrams of the unlabeled peptide was dissolved in 300 μ L of an aqueous (90% H₂O, 10% D₂O) micelle solution containing DPC-*d*₃₈ (80 mM). The pH was adjusted to 7.4 with a BIS-TRIS-*d*₁₉ and Benzoic Acid-*d*₅ (20 mM) buffer. All volumes were approximately 300 μ L and placed in Shigemi NMR tubes (Shigemi Inc. Allison Park, PA) with the tube glass susceptibility matched to the susceptibility of D₂O. Non-deuterated DPC was purchased from Avanti Polar Lipids (Alabaster, AL). Peptide was purchased from Global Peptide (Huntsville, AL) MS (ESI+, m/z) calculated for C₁₃₀H₁₇₉N₃₀O₂₈S 2642.06; found: 2641.7.

All NMR spectra were recorded at a temperature of 298.1 K on a four-channel Bruker AVANCE II spectrometer at a field strength B_0 of 18.79 T using a 5-mm inverse triple-resonance (TCI) ¹H/¹³C/¹⁵N, Z-axis PFG cryoprobe, and running TopSpin 2.0, pl 5 software. The ¹H, ¹³C and ¹⁵N chemical shifts for each residue were determined by analyzing ¹⁵N HSQC, DQF-COSY, TOCSY (31 and 61 ms), NOESY (80 and 200 ms), ¹³C HSQC and ¹³C HSQC-TOCSY spectra. D₂O exchange experiments were measured using 1D ¹H and 2D TOCSY spectra with Watergate to suppress a signal of residual HDO with the initial measurement in 15 minutes of D₂O addition and then continually scanning every 23 minutes for the first 19 hours. After 48 hours, spectra were then obtained every 23 minutes for 5 hours and then every hour for 11 hours. A final scan was acquired after 120 hours. NOE interactions between the peptide and micelle protons were determined using 2D NOESY (80 and 200 ms) spectra with peptide incorporated in a non-deuterated DPC micelle solution buffered at pH 7.4.

The relaxation rate constants R_1 ($R_1 = 1/T_1$), and R_2 ($R_2 = 1/T_2$) were determined from the cross-peak intensities of the corresponding 2D proton detected ¹⁵N HSQC like spectra.^{1, 2} For the R_1 and R_2 measurements, the spectra were recorded with delays T ; $T = 10, 35, 70, 110, 180, 300, 420, 570, 700, 850$ and 1100 ms and $T = 16, 32, 48, 64, 80, 96, 112, 128, 144, 160$ and 176 ms, respectively. To estimate the experimental error for R_1 and R_2 , the spectra measured with $T = 10$ and 420 ms, for the R_1

experiment and $T = 16$ and 96 ms for the R_2 experiment were measured twice. Relaxation delays of 2 seconds were used in all measurements. All spectra were obtained with spectral widths of 11161 Hz and 2270 Hz in the F_2 and F_1 domains respectively. Time domain data (t_2, t_1) were recorded as 2048 x 64 complex matrices with 160 scans per t_1 increment.

Calculations

The structure of the peptide was calculated and annealed using CYANA (LAS Systems Tokyo Japan).³ In the calculation, a total of 681 NOE signals were used with 561 being off-diagonal assignments and 460 were non-redundant NOE distance constraints. Of the non-redundant NOEs, 372, 171 and 18 were short, medium and long range assignments respectively with 120 being inter-residue NOEs. A total of 38 dihedral angle restraints were obtained from the ^{15}N , C_α , H_α and C_β backbone chemical shift values using the program TALOS.⁴ The peptide structure was calculated and annealed for seven iterations to provide 20 final structures.

The ^{15}N -spin-lattice relaxation rates R_1 , ^{15}N -spin-spin relaxation rates R_2 and ^{15}N heteronuclear NOE values were calculated for amide resonance signals using the CURVEFIT program (Palmer, Columbia).^{5,6} In the R_1 and R_2 measurements, cross peak intensities $I(t)$ were fit using two parameters (I_0, R_i), where:

$$I(t) = I_0 e^{-R_i t} \text{ where } i = 1, 2$$

The errors in the relaxation rates R_1 and R_2 were obtained from the root-mean-square deviations between the duplicated spectra. The errors are larger in the experiments in comparison to a 2 mM ^{15}N labeled peptide sample because the natural abundance of the ^{15}N isotope is very low, just 0.37%, so the effective sample concentration for all experiments involving ^{15}N nuclei is only 7.3 μM . The measured ^{15}N HSQC spectra used for calculation of R_1 , R_2 , and ^{15}N NOE values therefore exhibited rather low signal to noise ratio even when a cryoprobe was employed with several hundreds of scans per t_1

increment.

NOE values were obtained from the ratios of the corresponding cross peak intensities from the spectra recorded with presaturation and without presaturation during a relaxation delay of 3 seconds using the formula:

$$NOE = \frac{I_{sat}}{I_{unsat}}$$

The standard deviations of the NOE values were determined by the uncertainties of peak heights.

The NMR relaxation of ^{15}N atoms with directly attached hydrogens is dominated by dipolar interactions between ^{15}N and ^1H spins and by the ^{15}N chemical shift anisotropy (CSA).⁷ If cross correlation between these two relaxation mechanisms is negligible, the ^{15}N relaxation rates R_1 , R_2 and NOE can be expressed as linear combinations of the spectral densities, which are Fourier transforms of the autocorrelation functions that characterize molecular motions as follows:⁸

$$R_1 = d^2 / 4[J(\omega_H - \omega_N) + 3J(\omega_N) + 6J(\omega_H + \omega_N)] + c^2 J(\omega_N)$$

$$R_2 = d^2 / 8[4J(0) + J(\omega_H \omega_N) + 3J(\omega_N) + 6J(\omega_H) + 6J(\omega_H + \omega_N)] + c^2 / 6[4J(0) + 3J(\omega_N)] + R_{ex}$$

$$NOE = 1 + (d^2 / 4R_1)(\gamma_H / \gamma_N)[6J(\omega_H + \omega_N) - J(\omega_H - \omega_N)]$$

where

$$d = (\mu_0 h \gamma_H \gamma_N) / (8\pi^2 r_{NH}^3)$$

$$c = \omega_N / 3^{1/2} (\sigma_{\parallel} - \sigma_{\perp})$$

and μ_0 is the permeability of free space, h is Plank's constant, γ_H and γ_N are the gyromagnetic ratios of ^1H and ^{15}N respectively, r_{NH} is the N-H bond length (1.023×10^{-10} m), ω_H and ω_N are the Larmor frequencies of ^1H and ^{15}N respectively, $(\sigma_{\parallel} - \sigma_{\perp})$ is the ^{15}N CSA (-162 ppm) and $J(\omega)$ is the spectral density function. In an extended model free formalism,^{9,10} the spectral density is given by formula:

$$J(\omega) = \frac{2}{5} \left[\frac{S^2 \tau_m}{1 + (\omega \tau_m)^2} + \frac{(S_f^2 - S^2) \tau}{1 + (\omega \tau)^2} \right] = \frac{2}{5} S_f^2 \left[\frac{S_s^2 \tau_m}{1 + (\omega \tau_m)^2} + \frac{(1 - S_s^2) \tau}{1 + (\omega \tau)^2} \right]$$

in which

$$\tau = \frac{\tau_e \tau_m}{(\tau_e + \tau_m)}$$

τ_e is the effective correlation time characterizing internal motion, τ_m is the correlation time characterizing overall molecular tumbling, $S^2 = S_f^2 S_s^2$ is the square of the generalized order parameter that characterizes the amplitude of the internal motions and S_f^2 and S_s^2 are the squares of the order parameters for the internal motions on the fast and slow time scales, respectively.

The initial anisotropic tumbling rate τ_m (6.54 ns) for the MODELFREE program was calculated using the Stokes-Einstein relation:

$$\tau_m = \frac{1}{6D_{rot}} = \frac{\eta V_h}{kT} = \frac{4\pi a^3 \eta}{3kT} \quad \text{where} \quad D_{rot} = \frac{D_{\parallel}}{D_{\perp}} = \frac{2D_{zz}}{D_{xx} + D_{yy}}$$

where η is the viscosity, V_h is the volume of a sphere, k is the Boltzmann constant, T is the temperature in Kelvin and D_{rot} is the rotational diffusion constant for a sphere or a prolate or oblate

spheroid. The volume (0.5481 nm^3) of one DPC and number (55) of DPCs per micelle were calculated from previously published data,¹¹ the volume of the peptide was calculated using a program from Northwestern University (<http://www.basic.northwestern.edu/biotools/proteincalc.html>) and these values were used to calculate the total volume of the micelle/peptide complex (3.329 nm^3 , 21971.480 g/mol). The viscosity of water as a function of temperature was calculated from:

$$\eta = A \cdot 10^{\frac{B}{(T-C)}}$$

where A is $2.414 \times 10^{-5} \text{ Pa}\cdot\text{s}$, B is 247.8 K, C is 140 K and T is the experimental temperature between 273 and 373 K (<http://en.wikipedia.org/wiki/Viscosity>).

The relaxation rates R_1 , R_2 and NOE were analyzed using MODELFREE version 4.20.^{5,6} They were fitted to 5 dynamic models that differ in the number of parameters that can be adjusted. The variable parameters for individual models are *model 1*: the square of the generalized order parameter S^2 ; *model 2*: S^2 and the correlation time τ_c characterizing fast internal motions; *model 3*: S^2 and the chemical exchange term R_{ex} characterizing conformational exchange processes occurring on the μs - ms time scale; *model 4*: S^2 , R_{ex} , and the correlation time τ_c for fast internal motions; *model 5*: S^2 , S_f^2 the square of the order parameter for internal motions on the fast time scale, and the correlation time τ_c characterizing slow internal motions. The values of S^2 and S_f^2 span from zero, corresponding to isotropic internal motion, to one, corresponding to entirely restricted internal motion. The model selection protocol that was followed is described in a previous report.⁵

References

- (1) Kay, L. E.; Torchia, D. A.; Bax, A. *Biochemistry* **1989**, *28*, 8972-8979.
- (2) Farrow, N. A.; Muhandiram, R.; Singer, A. U.; Pascal, S. M.; Kay, C. M.; Gish, G.; Shoelson, S. E.; Pawson, T.; Forman-Kay, J. D.; Kay, L. E. *Biochemistry* **1994**, *33*, 5984-6003.
- (3) Guntert, P.; Mumenthaler, C.; Wuthrich, K. *J. Mol. Biol.* **1997**, *273*, 283-298.
- (4) Cornilescu, G.; Delaglio, F.; Bax, A. *J. Biomol. NMR* **1999**, *13*, 289-302.
- (5) Mandel, A. M.; Akke, M.; Palmer, A. G., III *J. Mol. Biol.* **1995**, *246*, 144-163.
- (6) Palmer, A. G., III; Rance, M.; Wright, P. E. *J. Am. Chem. Soc.* **1991**, *113*, 4371-4380.
- (7) Abragam, A., *Principles of Nuclear Magnetism*. ed.; Clarendon Press: Oxford, **1961**; p 599.
- (8) Cavanagh, J.; Fairbrother, W. J.; Palmer III, A. G.; Skelton, N. J., *Protein NMR Spectroscopy: Principles and Practice* ed.; Academic Press Inc. : San Diego, **1996**; p 587.
- (9) Clore, G. M.; Driscoll, P. C.; Wingfield, P. T.; Gronenborn, A. M. *Biochemistry* **1990**, *29*, 7387-7401.
- (10) Clore, G. M.; Szabo, A.; Bax, A.; Kay, L. E.; Driscoll, P. C.; Gronenborn, A. M. *J. Am. Chem. Soc.* **1990**, *112*, 4989-4991.
- (11) Lipfert, C. J.; Columbus, L.; Chu, V. B.; Lesley, S. A.; Doniach, S. *J. Phys. Chem.* **2007**, *111*, 12427-12438.