J|A|C|S
ARTICLES
Published on Web 10/03/2006

The Lipidated Membrane Anchor of Full Length N-Ras Protein Shows an Extensive Dynamics as Revealed by Solid-State NMR Spectroscopy

Guido Reuther,[†] Kui-Thong Tan,[‡] Alexander Vogel,[†] Christine Nowak,[‡] Klaus Arnold,[§] Jürgen Kuhlmann,[‡] Herbert Waldmann,[‡] and Daniel Huster*[,†,§]

Contribution from the Institute of Biotechnology, Junior Research Group "Structural Biology of Membrane Proteins", Martin Luther University Halle-Wittenberg, Kurt-Mothes-Str. 3, D-06120 Halle, Germany, Max Planck Institute of Molecular Physiology, Otto-Hahn-Str. 11, D-44227 Dortmund, Germany, and Institute of Medical Physics and Biophysics, University of Leipzig, Härtelstr. 16-18, D-04107 Leipzig, Germany

Received May 24, 2006; E-mail: daniel.huster@biochemtech.uni-halle.de

Abstract: Many proteins involved in signal transduction are equipped with covalently attached lipid chains providing a hydrophobic anchor targeting these molecules to membranes. Despite the considerable biological significance of this membrane binding mechanism for 5−10% of all cellular proteins, to date very little is known about structural and dynamical features of lipidated membrane binding domains. Here we report the first comprehensive study of the molecular dynamics of the C-terminus of membrane-associated full-length lipidated Ras protein determined by solid-state NMR. Fully functional lipid-modified N-Ras protein was obtained by chemical-biological synthesis ligating the expressed water soluble N-terminus with a chemically synthesized ^{2}H or ^{13}C labeled lipidated heptapeptide. Dynamical parameters for the lipid chain modification at Cys 181 were determined from static ^{2}H NMR order parameter and relaxation measurements. Order parameters describing the amplitude of motion in the protein backbone and the side chain were determined from site-specific measurements of ^{1}H-^{13}C dipolar couplings for all seven amino acids in the membrane anchor of Ras. Finally, the correlation times of motion were determined from temperature dependent relaxation time measurements and analyzed using a modified Lipari Szabo approach. Overall, the C-terminus of Ras shows a versatile dynamics with segmental fluctuations and axially symmetric overall motions on the membrane surface. In particular, the lipid chain modifications are highly flexible in the membrane.

Introduction

Membrane-associated proteins are dynamic molecules undergoing motions in the time window from picoseconds to seconds.[1−3] Apart from the well-known hydrophobic α-helix or β-barrel membrane anchors, ca. 5 to 10% of all cellular proteins bind to the bilayer by insertion of covalently attached lipid modifications.[4] This reversible membrane binding mechanism is particularly common among proteins involved in signal transduction where several protein−protein interactions occur at the plasma membrane.[5] Because of the two-dimensional diffusion at the membrane the likelihood for a membrane attached protein to interact with its downstream effectors is drastically increased as opposed to protein−protein interaction in the cytosol.

The GTP binding protein Ras is a central component of one of the major signal transduction cascades in biology. As it controls cell proliferation and differentiation, mutations in Ras may lead to uncontrolled cell growth and cancer.[6] In up to 30% of all human cancers mutated forms of Ras are found. The C-terminus of the molecule contains the membrane anchor. In N-Ras, two posttranslational lipid modifications, a farnesylation of Cys 186 and a palmitoylation of Cys 181, provide the molecule with sufficient hydrophobic character to insert into lipid bilayers. Although several NMR and X-ray structures of the soluble N-terminal domain of the different isoforms of Ras have been known for more than 10 years,[7−10] a first structural

[†] Martin Luther University Halle-Wittenberg.
[‡] Max Planck Institute of Molecular Physiology.
[§] University of Leipzig.

(1) Watts, A. *Biochim. Biophys. Acta* **1998**, *1376*, 297−318.
(2) Brüschweiler, R. Connections between NMR relaxation measurements and theoretical models of structural dynamics of biopolymers in solution. In *Nuclear Magnetic Resonance Probes of Molecular Dynamics*; Tycko, R., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1994; pp 301−334.
(3) Karplus, M.; McCammon, J. A. *Nat. Struct. Biol.* **2002**, *9*, 646−652.
(4) Schlesinger, M. J., Ed. *Lipid Modification of Proteins;* CRC Press: Boca Raton, FL, 1993.
(5) Casey, P. J. *Science* **1995**, *268*, 221−225.
(6) Wittinghofer, A.; Waldmann, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 4192−4214.
(7) Kraulis, P. J.; Domaille, P. J.; Campbell-Burk, S. L.; Van Aken, T.; Laue, E. D. *Biochemistry* **1994**, *33*, 3515−3531.
(8) Thapar, R.; Williams, J. G.; Campbell, S. L. *J. Mol. Biol.* **2004**, *343*, 1391−1408.
(9) Milburn, M. V.; Tong, L.; deVos, A. M.; Brünger, A.; Yamaizumi, Z.; Nishimura, S.; Kim, S. H. *Science* **1990**, *247*, 939−945.
(10) Pai, E. F.; Kabsch, W.; Krengel, U.; Holmes, K. C.; John, J.; Wittinghofer, A. *Nature* **1989**, *341*, 209−214.

10.1021/ja063635s CCC: $33.50

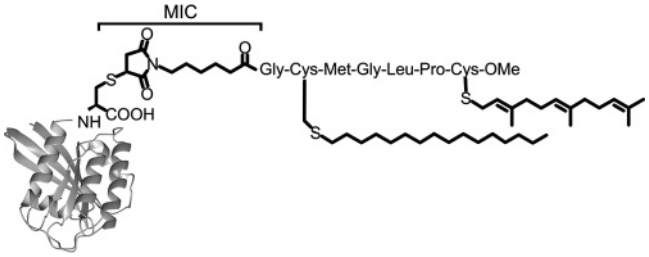
model of the membrane anchor at the C-terminus of the full-length Ras protein only became available recently.[11]

While structural data on membrane proteins is scarce, information about their mobility is even more limited. Since the vast majority of membrane protein structures was determined by diffraction, only very incomplete dynamics information is available from protein crystals. Usually, NMR methods are superior in characterizing the molecular motions in a large time window,[12] but the absence of the fast tumbling renders solution NMR analysis of membrane proteins difficult. However, great potential is attributed to solid-state NMR methods to investigate membrane bound proteins[13] in particular involving the magic-angle spinning (MAS) technique.[14−17] In addition to providing the sophisticated methodology required for measuring structural parameters, solid-state NMR spectroscopy has unique capabilities for studying the molecular dynamics of membrane proteins in their natural environment in a broad time window.[18]

Several studies have addressed the dynamics of the soluble N-terminus of Ras.[7,8,19] However, dynamic aspects of the membrane anchor of membrane bound Ras have never been studied. This part of the molecule not only attaches it to the bilayer but also determines the sorting of Ras into rafts or liquid crystalline domains.[20,21] Further, the structural homology between the different members of the Ras family is >90% in the GTP binding N-terminal domain but less than 15% in the C-terminal membrane anchor. However, the biological output of the highly homologous Ras proteins in vivo is very different; for instance K-Ras is more potent in activating Raf-1 than H-Ras, but less efficient to activate phosphoinositide-3-kinase.[20] The molecular mechanisms responsible for the different biological signaling of the Ras proteins must be related to structural and dynamical aspects of the C-termini of these molecules.

In this study we present the first report on the site-specific molecular dynamics of full-length membrane-associated lipid modified Ras protein. Selectively ^{2}H and ^{13}C labeled Ras protein was synthesized by a combination of chemical and recombinant strategies.[22] Molecular motions of the amino acids 180 to 186 and the lipid chain modification at Cys 181 are analyzed by ^{2}H and ^{13}C NMR methods. Thus, a comprehensive model of the molecular dynamics of the membrane anchor of a lipid modified membrane bound protein is obtained.

Scheme 1. Schematic Structure of the C-Terminal Lipid Modified N-Ras Protein[a]



[a] The sequence of the seven terminal amino acids investigated in this study is given. MIC: maleimidocaproyl.

Experimental Procedures

Materials. The phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-d_{54}-*sn*-glycero-3-phosphocholine (DMPC-d_{54}), and 1,2-dimyristoyl-d_{54}-*sn*-glycerol-3-phosphocholine-1,1,2,2-d_4−*N*,*N*,*N*-trimethyl-d_9 (DMPC-d_{67}) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Uniformly ^{13}C- and ^{15}N-labeled N^a-Fmoc-protected amino acids were purchased from Euriso-Top GmbH (Saarbrücken, Germany). Further reagents and solvents were obtained from Sigma-Aldrich.

Protein Synthesis. The synthesis of the N-Ras lipopeptide with a N-terminal maleimidocaproyl-function, a non-hydrolyzable hexadecylated cystein at the position of the palmitoylable cystein, and a farnesylation and carboxymethylation at the C-terminal cysteine has been described before.[22] The schematic structure of the lipopeptide is given in Scheme 1. Thus, the C-terminus is attached to the rest of the protein by maleinimide alkylation of a sylfhydryl residue rather than by an original peptide bond. Several lipopeptides with varying ^{13}C and/or ^{2}H labeling patterns with a hexadecyl and a farnesyl chain were synthesized.[23] Briefly, C-terminally truncated wild-type N-Ras (residues 1−181) was expressed in *E. coli* CK600K and purified via DEAE ion exchange chromatography and gel filtration. Coupling with isotopically labeled N-Ras lipopeptides was performed in stoichiometric amounts in 20 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$ supplemented with the detergent Triton X114. The detergent allowed for convenient separation of lipoprotein product and truncated educt after completion of the coupling reaction. After removal of Triton X114 from the N-Ras lipoprotein by another DEAE ion exchange chromatography, the lipoprotein was concentrated and adjusted to 20 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM DTE by size exclusion filtration in Amicon concentrators. All protein batches were analyzed by SDS-PAGE and MALDI-TOF mass spectrometry.

Sample Preparation. Large unilamellar vesicles were prepared by extrusion[24] in buffer (10 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$, pH 7.4). Aliquots of soluble Ras molecules were added to the liposome solution at a 1:150 protein to lipid molar ratio. After incubation at 37°C for 4 h the sample was ultracentrifuged at ∼90.000*g* for 10 h. Subsequently, the pellet was lyophilized and then hydrated to 35 wt % water content. After equilibration by several freeze−thaw cycles and gentle centrifugation the sample was transferred into 4 mm MAS rotors with Teflon insert.

Deuterium Solid-State NMR Spectroscopy. ^{2}H NMR spectra were acquired with a Bruker Avance 750 widebore NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at a resonance frequency of 115.1 MHz for ^{2}H. A single-channel solids probe equipped with a 5 mm solenoid coil was used. The ^{2}H NMR spectra were accumulated with a spectral width of ±250 kHz using quadrature phase

(11) Reuther, G.; Tan, K.-T.; Köhler, J.; Nowak, C.; Pampel, A.; Arnold, K.; Kuhlmann, J.; Waldmann, H.; Huster D. *Angew. Chem., Int. Ed Engl.* **2006**, *45*, 5387−5390.
(12) Palmer, A. G., III.; Williams, J.; McDermott, A. *J. Phys. Chem.* **1996**, *100*, 13293−13310.
(13) Torres, J.; Stevens, T. J.; Samso, M. *Trends Biochem. Sci.* **2003**, *28*, 137−144.
(14) Castellani, F.; van Rossum, B.; Diehl, A.; Schubert, M.; Rehbein, K.; Oschkinat, H. *Nature* **2002**, *420*, 98−102.
(15) Gröbner, G.; Burnett, I. J.; Glaubitz, C.; Choi, G.; Mason, A. J.; Watts, A. *Nature* **2000**, *405*, 810−813.
(16) Luca, S.; Heise, H.; Baldus, M. *Acc. Chem. Res.* **2003**, *36*, 858−865.
(17) Huster, D. *Prog. Nucl. Magn. Reson. Spectrosc.* **2005**, *46*, 79−107.
(18) Tycko, R. How does nuclear magnetic resonance probe moleculare dynamics. In *Nuclear Magnetic Resonance Probes of Molecular Dynamics*; Tycko, R., Ed.; Kluwer Academic Publishers: Dodrecht, The Netherlands, 1994; pp 1−26.
(19) Spoerner, M.; Herrmann, C.; Vetter, I. R.; Kalbitzer, H. R.; Wittinghofer, A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4944−4949.
(20) Parton, R. G.; Hancock, J. F. *Trends Cell Biol.* **2004**, *14*, 141−147.
(21) Nicolini, C.; Baranski, J.; Schlummer, S.; Palomo, J.; Lumbierres-Burgues, M.; Kahms, M.; Kuhlmann, J.; Sanchez, S.; Gratton, E.; Waldmann, H.; Winter, R. *J. Am. Chem. Soc.* **2006**, *128*, 192−201.
(22) Bader, B.; Kuhn, K.; Owen, D. J.; Waldmann, H.; Wittinghofer, A.; Kuhlmann, J. *Nature* **2000**, *403*, 223−226.
(23) Volkert, M.; Uwai, K.; Tebbe, A.; Popkirova, B.; Wagner, M.; Kuhlmann, J.; Waldmann, H. *J. Am. Chem. Soc.* **2003**, *125*, 12749−12758.
(24) Hope, M. J.; Bally, M. B.; Webb, G.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *812*, 55−65.

detection, a quadrupolar echo sequence[25] with two 4 μs $\pi/2$ pulses separated by a 60 μs delay, and a relaxation delay of 1.5 s. An inversion−recovery quadrupolar echo pulse sequence was used to measure the spin−lattice relaxation time. Details of the data analysis and the determination of smoothed order parameters[26] are described in the literature.[27] The Pake doublets have been assigned starting at the terminal methyl group, which exhibits the smallest quadrupolar splitting. The methylene groups were assigned consecutively according to their increasing quadrupolar splittings.

MAS NMR Experiments. All MAS NMR experiments were carried out on the Avance 750 spectrometer at resonance frequencies of 749.8 MHz for ^{1}H and 188.5 MHz for ^{13}C using a double-resonance MAS probe each equipped with a 4 mm spinning module. Chemical shifts were referenced relative to TMS.

^{13}C cross-polarization MAS spectra were acquired using a 4 μs ^{1}H 90° excitation pulse and a contact time of 0.7 ms. The strength of the ^{1}H-^{13}C dipolar couplings was measured using the constant time dipolar and chemical shift (DIPSHIFT) pulse sequence.[28] ^{1}H-^{1}H homonuclear decoupling was achieved with the frequency-switched Lee-Goldburg (FSLG) sequence.[29] Since the dipolar-induced signal decay is periodic, it was only necessary to acquire the signal over one rotor period in the indirect dimension. The time evolution of the C−H dipolar couplings in 2D DIPSHIFT experiments was simulated for one rotor period.[28] Simulations were performed for varying dipolar coupling strengths with powder averaging in 2° increments for the β and γ Euler angles.

^{13}C MAS T_1 and T_2 relaxation times were measured using standard pulse sequences. Typical ^{13}C $\pi/2$ pulse lengths were 5 μs. The temperature dependence of the relaxation times was simulated using a modified Lipari−Szabo approach[30] with an Arrhenius temperature dependence of the correlation times with $\tau_0 = 10^{-15}$ s.[12] It was further considered that two directly bonded hydrogens relax the ^{13}Cα spin of Gly 183. Anisotropic interactions are assumed both for the fast (f) and the slow (s) protein motions. The spectral density functions are given by[30]

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

where τ_i are the correlation times and S_i the order parameters, i = f, s, and $S = S_f \times S_s$.

Results

Lipid Chain Dynamics of Membrane Bound Ras Protein. Lipid-modified N-Ras proteins containing either one perdeuterated S-hexadecylated or one protiated S-hexadecylated Cys at position 181 and one S-farnesylated Cys at position 186 was synthesized according to known procedures.[22,23] The proteins with the perdeuterated or protiated hexadecyl chain modification are termed Ras-d_{33} and Ras, respectively. The Ras molecules were reconstituted into protiated DMPC or chain perdeuterated DMPC-d_{54} membranes and investigated by static ^{2}H NMR methods. To understand the molecular dynamics of the lipid modifications of membrane-associated Ras, we have compared the properties of the lipid chains in three different samples: (i) pure DMPC-d_{54}, (ii) DMPC-d_{54}/Ras at a 150:1 molar ratio, and (iii) DMPC/Ras-d_{33} at a 150:1 molar ratio. Thus, structural and dynamical information about the Ras hexadecyl chain attached to Cys 181 in comparison to the phospholipid chains can be extracted. Furthermore, this combination of samples allows determining the effect of the Ras protein on the bilayer properties of the host DMPC matrix.

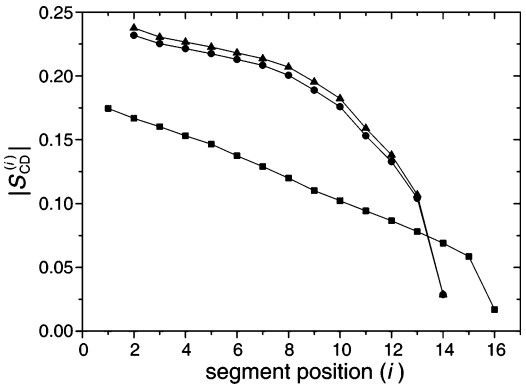


Figure 1. Profiles of the smoothed segmental order parameter $|S_{CD}^{(i)}|$ as a function of reverse chain segment index i for the hexadecyl chain of Ras and the myristoyl chains of DMPC, respectively. Data are shown for DMPC-d_{54} (●), DMPC-d_{54}/Ras (150:1 mol/mol) (▲), and DMPC/Ras-d_{33} (150:1 mol/mol) (■) at a buffer content of 35 wt % and 30 °C.

Table 1. Summary of Structural Results for DMPC-d_{54}, DMPC-d_{54}/Ras, and DMPC/Ras-d_{33} at 30 °C[a]

	$\langle A\rangle$/Å$^{2\,b}$	D_C/Å^c
DMPC-d_{54}	29.7 ± 0.4	12.9 ± 0.2
DMPC-d_{54}/Ras	29.5 ± 0.4	13.0 ± 0.2
DMPC/Ras-d_{33}	33.8 ± 0.8	13.0 ± 0.4

[a] The lipid protein molar ratio was 150:1. [b] Cross-sectional area of one hydrocarbon chain. [c] Volumetric hydrocarbon thickness.

From the ^{2}H NMR powder spectra of the respective samples, the segmental chain order parameters were determined using the de-Paking procedure.[31] Smoothed order parameter profiles showing the dependence of the order parameter on the position of the carbon segment in the acyl chain are presented in Figure 1. The segments are numbered consecutively starting at the carbonyl group of the lipid or the carbon directly connected to the sulfur atom of Cys 181 of Ras, respectively. Striking differences between the chain order parameters of DMPC-d_{54} and Ras-d_{33} are observed. Dramatically lower absolute order parameters are calculated from the narrower ^{2}H NMR spectrum of the Ras-d_{33} chains. This indicates that the hydrocarbon chains of the Ras protein occupy a substantially greater conformational space than the DMPC hydrocarbon chains. In contrast, the order profiles for DMPC-d_{54} in the presence and in the absence of Ras are very similar.

From the ^{2}H NMR data, the average area per hydrocarbon chain $\langle A\rangle$ and the thickness of the hydrocarbon region D_C can be calculated.[27,32] Table 1 reports these values. The hydrocarbon thickness for DMPC-d_{54} is in good agreement with X-ray results.[33] The slightly higher absolute magnitudes of the order parameters of DMPC-d_{54} in the presence of Ras indicate a slightly thicker hydrocarbon core, so that the interfacial area per chain is somewhat smaller compared to pure DMPC-d_{54}.

(25) Davis, J. H.; Jeffrey, K. R.; Bloom, M.; Valic, M. I.; Higgs, T. P. *Chem. Phys. Lett.* **1976**, *42*, 390−394.
(26) Lafleur, M.; Fine, B.; Sternin, E.; Cullis, P. R.; Bloom, M. *Biophys. J.* **1989**, *56*, 1037−1041.
(27) Vogel, A.; Katzka, C. P.; Waldmann, H.; Arnold, K.; Brown, M. F.; Huster, D. *J. Am. Chem. Soc.* **2005**, *127*, 12263−12272.
(28) Hong, M.; Gross, J. D.; Griffin, R. G. *J. Phys. Chem.* **1997**, *101*, 5869−5874.
(29) Bielecki, A.; Kolbert, A. C.; Levitt, M. H. *Chem. Phys. Lett.* **1989**, *155*, 341−345.
(30) Clore, G. M.; Szabo, A.; Bax, A.; Kay, L. E.; Driscoll, P. C.; Gronenborn, A. M. *J. Am. Chem. Soc.* **1990**, *112*, 4989−4991.
(31) Sternin, E.; Bloom, M.; MacKay, L. *J. Magn. Reson.* **1983**, *55*, 274−282.
(32) Petrache, H. I.; Dodd, S. W.; Brown, M. F. *Biophys. J.* **2000**, *79*, 3172−3192.
(33) Koenig, B. W.; Strey, H. H.; Gawrisch, K. *Biophys. J.* **1997**, *73*, 1954−1966.

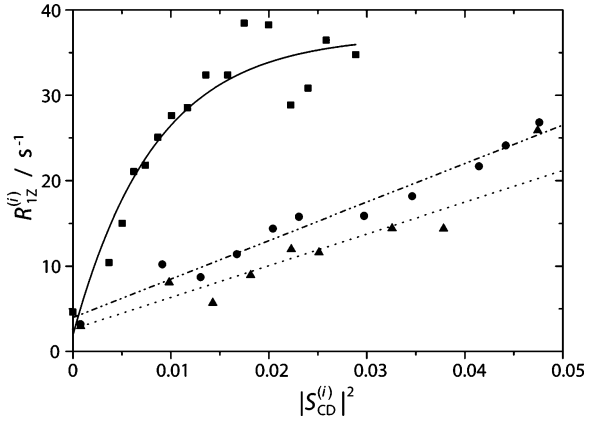


Figure 2. Dependence of the $R_{1Z}^{(i)}$ relaxation rates on the corresponding order parameter squared for DMPC-d_{54} (●), DMPC-d_{54}/Ras (150:1 mol/mol) (▲), and DMPC/Ras-d_{33} (150:1 mol/mol) (■) at 115.1 MHz (17.6 T). Lines are drawn to guide the eyes. Assuming the relaxation is governed by order fluctuations, the lipid modification of Ras is much more flexible than that of DMPC-d_{54}.

For Ras-d_{33} in a DMPC matrix the hydrocarbon thickness is very close to the values for DMPC-d_{54}, while the average area per hydrocarbon chain is more than 4 Å^2 larger. This is in good agreement with former studies on a lipidated Ras peptide (residues 180−186), which also showed the Ras and DMPC chains to match in their lengths.[27,34] The large cross-sectional area of the Ras chain provides space for the peptide backbone that occupies a substantial area in the lipid-water interface of the membrane. The free space underneath the peptide backbone in the membrane core is "filled" by the hydrophobic peptide side chains (Leu and Met[34]) and by the large amplitude motions of the Ras lipid chains covalently bound to the Cys residues. Thus, the polar protein backbone forms an "umbrella" shielding the hydrophobic side chains and lipid modifications of Ras from the water.[35]

To provide dynamics information of the chain modification at Cys 181 of Ras, ^{2}H NMR spin−lattice relaxation time (T_{1Z}) measurements were performed for the various samples studied. The inverse of T_{1Z} defines the spin−lattice relaxation rate R_{1Z}. The relaxation rates for the Ras-d_{33} hexadecyl chain are generally higher than for DMPC-d_{54} (data not shown). In previous analyses of pure phospholipid membranes, it has been found that the relaxation rate often exhibits a linear dependence on the square of the order parameter.[36] While the physical reason for this interesting behavior is still under discussion, it has been shown to be a common feature for disaturated phospholipids. The square-law plots for DMPC-d_{54} in the presence and in the absence of the Ras as well as for Ras-d_{33} in a DMPC matrix are shown in Figure 2. For DMPC-d_{54} in the presence as well as in the absence of Ras protein, the dependence is linear. However, the $R_{1Z}^{(i)}$ versus $|S_{CD}^{(i)}|^2$ plot for the Ras-d_{33} hexadecyl chain departs from the linear dependence and shows a bent shape with a steeper slope.

These R_{1Z} versus $|S_{CD}|^2$ plots report the elastic properties of the lipid chains in a membrane.[37] In softer materials, the acyl chains are undergoing large amplitude motions, which is expressed in a steeper slope in the square-law plots with a nonlinear dependence. This is exactly the behavior observed for the Ras hexadecyl chain modification at Cys 181. This Ras lipid modification is not only more loosely packed as expressed by low order parameters, it also appears to be highly flexible and dynamic, undergoing motions with large angular amplitudes. A material made of such highly mobile chains would be soft and very elastic. Interestingly, the lipid chains of the host matrix show a somewhat shallower slope in the square-law plots, which means, that the elasticity of the host membrane is actually slightly reduced. This again underlines that the changes in the membrane caused by the insertion of the Ras protein are mostly compensated for by the mobility of the Ras chains rather than by the host matrix.

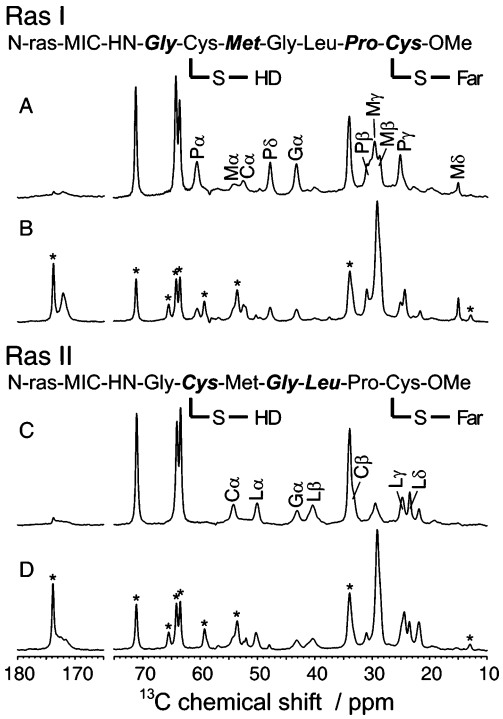


Figure 3. Proton decoupled 188.5 MHz ^{13}C CP MAS NMR (A, C) and directly polarized ^{13}C MAS Bloch decay (B, D) spectra of Ras bound to DMPC-d_{67} vesicles (1:150 molar ratio) at a buffer content of 35 wt % and a temperature of 30 °C. The labeling scheme of the Ras proteins and the signal assignments are also given.[11] Labeled amino acids are shown in bold italics. The asterisks indicate signals from the DMPC matrix.

Molecular Dynamics of the C-Terminus of Membrane Bound Ras Protein. In addition to the dynamics information for the Ras lipid modification at Cys 181, we studied the backbone and side chain molecular mobility of the seven C-terminal amino acids of Ras by ^{13}C MAS NMR spectroscopy. To avoid overlapping of the NMR signals, two complementary ^{13}C labeled Ras proteins were synthesized (termed Ras I and Ras II, see Figure 3). The chemical synthesis of lipidated Ras peptides prior to coupling of the expressed N-terminus allows introducing selective NMR labels into the protein segment of interest. In Figure 3, both directly- (Bloch decay) and cross-polarized (CP) ^{13}C MAS NMR spectra are shown for each sample. Most of the phospholipid signals are suppressed by using an almost completely perdeuterated DMPC-d_{67} matrix. Only the nondeuterated glycerol backbone resonances from the phospholipids are detected in these spectra. In addition,

(34) Huster, D.; Vogel, A.; Katzka, C.; Scheidt, H. A.; Binder, H.; Dante, S.; Gutberlet, T.; Zschörnig, O.; Waldmann, H.; Arnold, K. *J. Am. Chem. Soc.* **2003**, *125*, 4070−4079.
(35) Huang, J.; Feigenson, G. W. *Biophys. J.* **1999**, *76*, 2142−2157.
(36) Brown, M. F.; Ribeiro, A. A.; Williams, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4325−4329.
(37) Brown, M. F.; Thurmond, R. L.; Dodd, S. W.; Otten, D.; Beyer, K. *J. Am. Chem. Soc.* **2002**, *124*, 8471−8484.

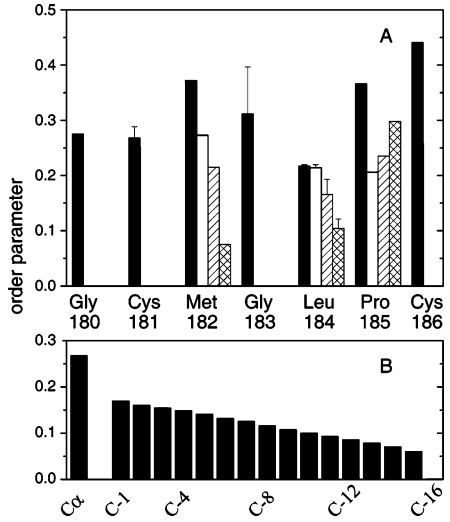


Figure 4. ^{1}H-^{13}C order parameters of the membrane bound C-terminus of Ras. (A) Order parameters for the protein backbone and side chains are given for the seven terminal residues. Filled bars correspond to the order parameters of Cα sites, open bars to the Cβ, hatched bars to the Cγ, and crossed bars to the Cδ sites. (**B**) Combined plot of the order parameters of Cys 181 starting at the Cα site and continuing into the lipid chain membrane anchor. The hexadecyl chain order parameters were taken from Figure 1.

the highly mobile headgroup and chain end signals of DMPC-d_{67} are seen in Bloch decay spectra because the dipolar ^{2}H-^{13}C dipolar couplings are averaged out by motions. The protein signal assignment was obtained previously.[11] The typical line width of these ^{13}C signals is on the order of 1 ppm.

The good resolution of the 1D ^{13}C NMR spectra allows determining the strength of the ^{1}H-^{13}C dipolar couplings in a DIPSHIFT experiment.[28] Fast anisotropic molecular motions partially average these couplings. Therefore, the amplitude of the motion of the bond vectors can be assessed from the order parameter defined by the ratio of the motionally averaged and full dipolar coupling.[12] ^{1}H-^{13}C order parameters of the terminal amino acids of membrane-associated lipidated Ras protein are shown in Figure 4. The absolute values for these order parameters are relatively low indicating large amplitude motions in the C-terminus of membrane bound Ras. A monotonic decrease of the order parameters along the side and lipid chains is observed indicating that the amplitude of motions increases along the chain. Only for Pro 185, order parameters decrease for Cβ and increase again for Cγ and Cδ as expected for the five-membered ring that folds back to the main chain. There is no conclusive trend in the amplitude of the backbone motions of the membrane bound Ras protein. It is interesting that the two lipid modified Cys residues exhibit very different order parameters suggesting that the farnesylated Cys 186 is more rigid than the hexadecylated Cys 181.

Finally, it is important to know the correlation times of the molecular motions studied. To this end, we measured ^{13}C MAS NMR T_1 and T_2 relaxation times as a function of temperature for membrane bound ^{13}C-Gly-183-Ras protein, shown in Figure 5. With increasing temperature, T_1 relaxation times decrease while T_2 relaxation times increase as typical for samples exhibiting molecular motions that are not in the extreme narrowing limit. To calculate correlation times for the backbone motions of membrane bound Ras, a modified Lipari−Szabo formalism was used.[30] Since membrane binding restricts the mobility of the Ras protein, the spectral density functions of

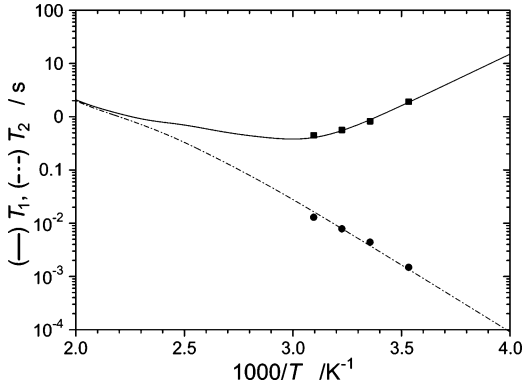


Figure 5. Experimental and simulated relaxation times vs inverse temperature data for the ^{13}Cα signal of Gly 183 of membrane bound Ras protein. The symbols refer to the experimental T_1 (■) and T_2 (●) relaxation times, and the lines are the best fit simulation using a modified Lipari−Szabo approach.

Table 2. Correlation Times for the Slow (τ_s) and Fast (τ_f) Motions of Gly 183 in Membrane-Associated Lipidated Ras Protein[a]

temperature/K	τ_s/ns	τ_f/ns
283	864.0 ± 22.2	7.4 ± 0.3
298	306.7 ± 7.9	3.3 ± 0.1
310	143.9 ± 3.7	1.9 ± 0.1
323	67.6 ± 1.6	1.04 ± 0.05

[a] Data given are determined from the measured temperature dependence of the T_1 and T_2 relaxation times using a modified Lipari−Szabo approach. The order parameters are $S_s = 0.445 \pm 0.010$ and $S_f = 0.70 \pm 0.01$ and the activation energies for the dynamical processes are $E_{A,s} = (33.7 \pm 0.8)$ kJ/mol and $E_{A,f} = (25.9 \pm 0.6)$ kJ/mol.

the modified Lipari−Szabo approach contained one anisotropic term for the overall and one for the internal protein motions. The anisotropy is represented in an order parameter for the fast internal (S_f) and slower (S_s) overall motions of membrane-associated Ras. The product of both represents the order parameter measured in the DIPSHIFT experiments before. For the temperature dependence of the correlation times, an Arrhenius behavior was assumed. Therefore, the number of unknowns is reduced to three (one order parameter and the two activation energies for the temperature dependence of each correlation time). These parameters could be determined from the experimentally measured temperature dependence of the relaxation times. The results of the analysis are shown in Figure 5 and Table 2. Depending on temperature, the correlation times are between ~70 and 900 ns for the overall and between ~1 and 7 ns for the internal motions. The order parameters for these dynamic processes are 0.445 and 0.70, respectively, and $S_{DIPSHIFT} = S_f \times S_s = 0.312$.

Discussion

The main goal of this study was the elucidation of the complex dynamics of the lipidated C-terminus of membrane-associated Ras protein. Although Ras is biochemically (but not biologically) active in the absence of this membrane anchor, it is known that all members of the Ras family interact with the same effectors in vitro but generate distinct outputs in vivo. This suggests that the biological difference of the Ras isoforms is most likely imparted by their C-termini. According to the recent structural model of the Ras membrane anchor, the lipid modifications as well as the hydrophobic side chains insert into the bilayer core while the backbone is located in the membrane

interface.[11,34,38] Such a structural assembly in the highly mobile membrane raises questions about the molecular dynamics of the membrane anchor of Ras with regard to the stability of the membrane insertion and the molecular processes involved in the interaction with downstream effectors in the signaling cascade. Several aspects of the C-terminal dynamics of Ras are worth discussing with regard to their biological significance.

It was calculated from the ^{2}H NMR results that the lipid modification at Cys 181 adjusts its length to that of the host membrane. Instead of the membrane adjusting to the longer protein chain by local curvature, which costs free energy, the chain inserts into the hydrophobic membrane interior with almost no energy cost, thereby largely retaining its configurational entropy and leaving the host matrix relatively unchanged. This requires the protein chain to be flexible in order to insert into a fluid membrane that is characterized by a large amount of molecular disorder. However, first preliminary data on Ras partitioning into POPC membranes have shown that the order parameters of the Ras hexadecyl chains are very similar in both lipid matrixes despite the much different order parameters of the POPC and DMPC host membranes. This may indicate that the matching between the Ras and the DMPC chain lengths happened by chance and the low order parameter of the Ras lipid modifications result from the insertion of the protein backbone and side chains into the lipid water interface of the membrane. Thereby, the phospholipids molecules are spaced apart, which decreases the lateral pressure in the membrane underneath. The lipid chain modifications of Ras can fill the volume below the protein backbone by assuming very low order parameters, which means high amplitude motions for these chains. Therefore, the matching of the length of the Ras hydrocarbon chains with those of the phospholipid matrix is a result of energetically favorable hydrophobic contacts between the Ras and the DMPC chains as well as the accommodation of the interfacial area of the Ras chains to the area that the peptide backbone occupies in the membrane interface. While both effects are energetically favorable, the example of Ras partitioning into the POPC matrix may indicate that the low order parameters of the Ras lipid chains are mostly the result of additional area owing to the insertion of the protein backbone.

The dynamic properties of this lipid chain completely differ from that of the phospholipid membrane. Interestingly, the dynamic properties of the Ras lipid chain at Cys 181 resemble those of phospholipid chains in very soft membranes. Such bilayers are characterized by very flexible and mobile acyl chains described by low order parameters and large amplitude motions.[37] In other words, the lipid chain of Cys 181 is characterized by a high amount of flexibility. Previously, we investigated the structure and dynamics of the lipid modification of the lipid modified Ras heptapeptide.[27] Interestingly very similar structural and dynamical features of the lipid chain were found for the peptide indicating that the lipid chain modifications of Ras may move relatively independent of the rest of the protein. The relaxation rates determined for the hexadecyl chain of the Ras protein are about 10% higher, which can be understood by considering the molecular mass difference between peptide and protein, which results in somewhat slower motions of the latter.

The lipid modification of Cys 181 is the second posttranslational event for the N-Ras protein. First, Cys 186 is farnelsylated but the hydrophobic energy arising from this single lipid chain is not sufficient to permanently anchor the molecule in the membrane.[39] From the second lipid modification at Cys 181 the molecule may gain a maximum of −35 kJ/mol in hydrophobic binding energy.[40] Through the high flexibility of this lipid chain, which includes the possibility of occasional chain upturns, this binding energy could be significantly reduced and may even approach kT.[41,42] Although an immobile lipid chain would provide maximal hydrophobic interactions with the membrane, the entropic contribution to the free energy of protein binding would be unfavorable. For stable membrane insertion, two lipid modifications are necessary.[39,43] While the farnesyl moiety appears to be more rigid as inferred from the higher order parameter of Cys 186, the highly flexible and mobile Ras hexadecyl chain modification contributes sufficient hydrophobic binding energy but also configurational entropy, which both reduces the free energy of the membrane bound Ras protein and contributes to stable membrane insertion. According to this model, the branched farnesyl chain provides the more rigid membrane anchor while the second flexible chain modification can contribute both hydrophobic energy but also favorable configurational entropy and therefore reduce the free energy of the membrane ensemble.

In generalizing these findings, it will further be necessary to study the dynamic properties of the Ras chain modifications in more rigid membranes such as DPPC and DPPC/cholesterol mixtures. From our preliminary POPC results it appears that the flexible and highly dynamic Ras chain modification is an intrinsic property and not related to the elastic properties of the host membranes. Experiments in more rigid membrane matrixes are currently progress in our laboratory.

The motional amplitudes of the seven terminal amino acids of the membrane-associated Ras protein were determined using ^{13}C CP MAS NMR experiments. It is particularly noteworthy that the order parameter of farnesylated Cys 186 is about 60% higher than that of hexadecylated Cys 181. Considering the high degree of flexibility of the chain of Cys 181, this would suggest that the farnesyl modification of Cys 186 is relatively rigid. Currently, very little is known about the mobility of farnesyl moieties in membranes; however, molecular dynamics and NMR results suggest that they exist primarily in an extended conformation[44] and have no effect on the lipid chain order in phospholipids membranes.[45] The higher order of Cys 186 compared to Cys 181 suggests that the farnesyl chain may not show the high degree of flexibility that was observed for the hexadecyl chain. From its branched structure, the farnesyl chain represents a barbed hook that may more stably insert into the membrane than the very flexible hexadecyl chain.

At first glance surprising, the protein order parameters are relatively low compared to other membrane proteins.[46−48]

(38) Huster, D.; Kuhn, K.; Kadereit, D.; Waldmann, H.; Arnold, K. *Angew. Chem., Int. Ed.* **2001**, *40*, 1056−1058.

(39) Dudler, T.; Gelb, M. H. *J. Biol. Chem.* **1996**, *271*, 11541−11547.
(40) Tanford, C. *The Hydrophobic Effect: Formation of Micelles and Biological Membranes;* John Wiley & Sons: New York, 1980.
(41) Pool, C. T.; Thompson, T. E. *Biochemistry* **1998**, *37*, 10246−10255.
(42) Peitzsch, R. M.; McLaughlin, S. *Biochemistry* **1993**, *32*, 10436−10443.
(43) Shahinian, S.; Silvius, J. R. *Biochemistry* **1995**, *34*, 3813−3822.
(44) Zahn, T. J.; Eilers, M.; Guo, Z.; Ksebati, M. B.; Simon, M.; Scholten, J. D.; Smith, S. O.; Gibbs, R. A. *J. Am. Chem. Soc.* **2000**, *122*, 7153−7164.
(45) Rowat, A. C.; Davis, J. H. *Biochim. Biophys. Acta* **2004**, *1661*, 178−187.
(46) Huster, D.; Xiao, L.; Hong, M. *Biochemistry* **2001**, *40*, 7662−7674.
(47) Colnago, L. A.; Valentine, K. G.; Opella, S. J. *Biochemistry* **1987**, *26*, 847−854.

However, it should be recalled that the membrane anchor of Ras is not folded into a regular secondary structure.[11] Further, the order parameters need to be related to the time scale on which they are measured. For the amino acid order parameters determined from dipolar couplings all motions with correlation times shorter than ~40 μs contribute. Therefore, for a complete discussion of the different modes of mobility in the polypeptide chain knowledge about the correlation times of motions is required.

We determined these correlation times from the analysis of ^{13}C relaxation rates of Gly 183 in membrane-associated Ras. In general, in solids the relaxation rates exhibit a complicated angular dependence that is not completely averaged out by MAS.[49] However, considering the low order parameters of membrane bound Ras protein a Lipari−Szabo approach should provide a reasonable approximation for the dynamical behavior of the protein. This is supported by the obvious temperature dependence of the T_2 relaxation times of Ras, which is typically not observed in rigid solids. In the original Lipari−Szabo approach, the molecular dynamics is explained by a correlation function for the (isotropic) overall tumbling of the protein and an (anisotropic) internal motion. The absence of the isotropic tumbling of membrane bound proteins leads to a simplified spectral density function where the overall correlation time approaches infinity and the relaxation is solely explained by internal motions.[50] However, such a naive model does not describe the temperature dependence of the relaxation data determined in our study. This suggests that also somewhat slower motions of the lipidated C-terminus of the Ras protein must contribute to the relaxation. In contrast to the original Lipari−Szabo formalism for the membrane-associated Ras protein these motions would be anisotropic. These can be considered in a modified model, where anisotropy is assumed for both internal and overall mobility expressed by the respective order parameters.[30] This model excellently agreed with the measured relaxation rates. The order parameter for the fast motion of the Gly 183 Cα−Hα bond vector is 0.70 and for the slow motions of the membrane anchor it is 0.445.

On the basis of this formalism, the molecular mobility of the membrane-associated Ras protein can be disentangled into a fast segmental and a slower overall process. At 37 °C the fast bond vector fluctuations of Gly 183 occur with a correlation time of 1.9 ns and an amplitude of ~40°. Such segmental fluctuations are quite common for amino acids in somewhat flexible loop structures of soluble proteins. This agrees with the structural model of membrane-associated Ras protein that showed no regular secondary structure.[11] On the other hand, the membrane anchor of Ras inserts into the lipid water interface of the membrane[34,38] where it experiences a highly dynamic structural assembly of lipids and water molecules.[51] This suggests that membrane binding of Ras stabilizes the structure of its membrane anchor presumably by hydrogen bonds between the protein backbone and phospholipids segments in the membrane interface.

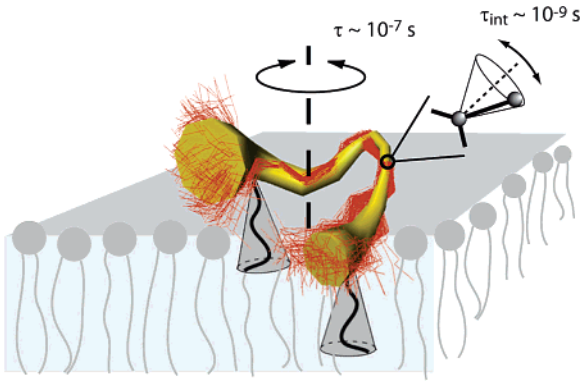


Figure 6. The cartoon illustrates the main results of this study (not to scale). The lipid modified C-terminus of the membrane bound N-Ras protein is highly mobile on several time scales. The protein backbone is shown as the 200 lowest energy structures determined recently (orange).[11] The flexibility of the structure is represented by the yellow tube. Fast segmental motions of the protein backbone and side chains occur with correlation times in the nanosecond range. The entire C-terminus of Ras undergoes axially symmetric motions on the membrane surface with correlation times of several 100 ns. The lipid modifications are also highly mobile and flexible in the membrane. The N-terminus and the side chains of the Ras protein are omitted for clarity. The protein structure was drawn in MolMol.[53]

In addition to the segmental mobility, the membrane anchor of Ras undergoes slower motions with large amplitudes (order parameter of 0.445). The correlation time for this motion is ~140 ns at 37 °C. From the molecular structure and the binding model of Ras it is reasonable to assume axially symmetric motions for Ras. This may also explain the good resolution found in the ^{1}H MAS NMR spectra of membrane-associated Ras proteins.[11] For molecules undergoing fast axially symmetric motions the dipolar broadening only depends on the orientation of the local bilayer normal relative to the external magnetic field. Thus, the homogeneous dipolar broadening is converted into an inhomogeneous broadening, which can be averaged out by MAS even if the rotational frequency is lower than the broadening.[52]

In summary, the C-terminus of membrane-associated full length Ras protein shows a versatile dynamics (Figure 6). The segmental motions of the protein backbone are similar to those of loop structures of soluble proteins. We suggest that binding to the lipid membrane stabilizes the structure of the C-terminus of Ras. Further, the membrane anchor of Ras undergoes slower motions with axial symmetry, which may apply to the entire protein. In fact, a recent solution NMR study of full-length farnesylated Ras also indicated that the motions of the N-terminal domain are influenced by the lipidated C-terminus in solution.[8] The lipid chain modification at Cys 181 is highly mobile in the liquid-crystalline membrane.

Acknowledgment. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Grants HU 720/5-2 and SFB 642).

JA063635S

(48) Saitô, H.; Tsuchida, T.; Ogawa, K.; Arakawa, T.; Yamaguchi, S.; Tuzi, S. *Biochim. Biophys. Acta* **2002**, *1565*, 97−106.
(49) Giraud, N.; Blackledge, M.; Goldman, M.; Bockmann, A.; Lesage, A.; Penin, F.; Emsley, L. *J. Am. Chem. Soc.* **2005**, *127*, 18190−18201.
(50) Cole, H. B. R.; Torchia, D. A. *Chem. Phys.* **1991**, *158*, 271−281.
(51) White, S. H.; Ladokhin, A. S.; Jayasinghe, S.; Hristova, K. *J. Biol. Chem.* **2001**, *276*, 32395−32398.
(52) Davis, J. H.; Auger, M.; Hodges, R. S. *Biophys. J.* **1995**, *69*, 1917−1932.
(53) Koradi, R.; Billeter, M.; Wüthrich, K. *J. Mol. Graph.* **1996**, *14*, 51−32.