Membrane Proteins

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Solid-state NMR Study of the YadA Membrane-Anchor Domain in the Bacterial Outer Membrane

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Abstract: *MAS-NMR was used to study the structure and dynamics at ambient temperatures of the membrane-anchor domain of YadA (YadA-M) in a pellet of the outer membrane of E. coli in which it was expressed. YadA is an adhesin from the pathogen Yersinia enterocolitica that is involved in interactions with the host cell, and it is a model protein for studying the autotransport process. Existing assignments were sucessfully transferred to a large part of the YadA-M protein in the E. coli lipid environment by using ^{13}C-^{13}C DARR and PDSD spectra at different mixing times. The chemical shifts in most regions of YadA-M are unchanged relative to those in microcrystalline YadA-M preparations from which a structure has previously been solved, including the ASSA region that is proposed to be involved in transition-state hairpin formation for transport of the soluble domain. Comparisons of the dynamics between the microcrystalline and membrane-embedded samples indicate greater flexibility of the ASSA region in the outer-membrane preparation at physiological temperatures. This study will pave the way towards MAS-NMR structure determination of membrane proteins, and a better understanding of functionally important dynamic residues in native membrane environments.*

Adhesion to host tissues is the first step in most bacterial infections, and bacteria have evolved a wide range of adhesins for this purpose.[1] Trimeric autotransporter adhesins (TAAs)[2] are a subfamily of type V secretion systems[3] that display a unique mechanism of "autotransport". *Yersinia* adhesin A (YadA) is a prototypical member of the TAA family and, like other family members, it forms a stable trimeric pore inside the outer membrane (OM), which functions as the translocation pore for its extracellular adhesive domain.[4] The transmembrane β-barrel domain is the only domain that is conserved throughout the TAA family, and it is sufficient for the autotransport mechanism.[5]

The structural and functional properties of membrane proteins (MPs) depend on the interactions with neighboring molecules in their native environment.[6] Membrane proteins are routinely studied in artificially prepared proteoliposomes to create a native-like environment and to achieve high resolution and sensitivity in solid-state NMR (ssNMR) spectra.[7] However, such preparations require considerable effort and do not reflect the membrane asymmetry of natural systems such as the OM of Gram-negative bacteria, in which the outer leaflet contains lipopolysaccharides while the inner leaflet is composed mostly of phospholipids. There are only a handful of solid-state MAS NMR studies of proteins within their native membranes.[8]

The original ssNMR structure of YadA-M was solved from a microcrystalline sample, and a stretch of four residues (Ala, Ser, Ser, Ala) in the translocation domain was found to be highly flexible based on its nonhelical NMR chemical shifts, reduced dipolar couplings, higher random-coil index, and predicted low-order parameter values.[9] This ASSA region is thought to form a short-lived hairpin in the transition state of the autotransport process, in line with the now generally accepted hairpin model of autotransport.[3,9b]

The aim of this study was to analyze the structure and dynamics of YadA-M in the *E. coli* OM, in which it was expressed without further purification, and to identify possible differences between the different sample preparations, since recent studies have shown structural differences for some proteins depending on the membrane mimetic or detergent employed.[10] We further present the partial chemical-shift assignment of YadA-M, provide simple strategies to distinguish between protein and lipid signals, and give important insights into the dynamics of the protein domain in the lipid bilayer. While no dramatic structural changes are observed between microcrystalline YadA-M and YadA-M in the OM, crystal contacts are lost and the functionally relevant ASSA region is predicted to be more flexible compared to the microcrystalline sample. This study demonstrates that membrane-embedded, unpurified, selectively labeled OM proteins are amenable to MAS-NMR structural and dynamic studies under physiological conditions.

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We used a comparatively simple strategy to prepare uniformly and selectively labelled YadA-M samples in the *E. coli* OM to aid in resonance assignment. In brief, outer membranes were isolated by differential centrifugation steps after protein expression and transferred directly to the ssNMR rotors (see the Supporting Information for details). The amount of protein present in the rotors was estimated to be 10 mg at approximately 70 % purity (5–7 mg of YadAM) in a pellet of approximately 50 mg wet weight. In contrast to previous studies on MPs in membrane patches by using ssNMR,[11] we measured under physiological conditions (room temperature) to retain essential information on protein dynamics. This method should be generally applicable to other MPs that can be overexpressed in the *E.coli* OM. A selectively labeled sample was also prepared with C13-labeled residues Gly, Val, Ala, and Ser (GVAS), which are present in all three secondary-structure elements of YadA-M.[9b] Importantly, the ASSA region is covered by this labeling Scheme (Figure S1 in the Supporting Information). SDS polyacrylamide gel electrophoresis (PAGE) of the extracted OM shows that approximately 70 % of the protein present is YadA-M. This results in higher NMR signals from the YadA-M compared to other OM proteins (Figure S2).

To unambiguously assign YadA-M, it is necessary to distinguish between the protein and lipid signals. For this, we adopted a threefold strategy. 1) Comparison of 1D ^{1}H-^{13}C spectra from YadA-M in microcrystalline and membrane environments gives a good indication of the lipid chemical shift values (Figure 1 A and Figure S3) since the lipid signals strongly differ in intensity and chemical shift compared to the protein signals. In particular, additional signals appear in the window $\delta = 65$–110 ppm that can be unambiguously attributed to lipids. 2) Since GVAS and uniformly ^{13}C-labeled YadA-M samples were prepared under the same conditions, the lipids should show similar cross-peaks in both samples, such that spectra comparison aids in identifying lipid cross-peaks. For instance, an overlay of spectra of the selectively and uniformly ^{13}C-labeled samples (Figure 1 B and Figure S4) shows that the uniformly labeled sample gives a number of new peaks in the spectral region around 40-62 ppm that can unambiguously be attributed to YadA-M. The membrane-bound YadA-M lines in the aliphatic ($\delta = 25$–35 ppm, 43–60 ppm) and carbonyl ($\delta = 170$–180 ppm) regions overlap closely with those of microcrystalline YadA-M and hence can also be identified as protein signals (Figure 2 and Figure S5). The region around $\delta = 25$–40 ppm is typical for lipid signals, therefore we left the correlations in this region unassigned owing to the high degree of overlap. 3) An additional line of evidence comes from the gel-to-liquid phase transition in the membrane preparations.[12] Peaks from lipids underwent dramatic changes in intensity with increasing temperature while the protein signals remained at constant intensity, so comparison of 1D spectra at different temperatures helps to distinguish protein and lipid signals (Figure 1 C).

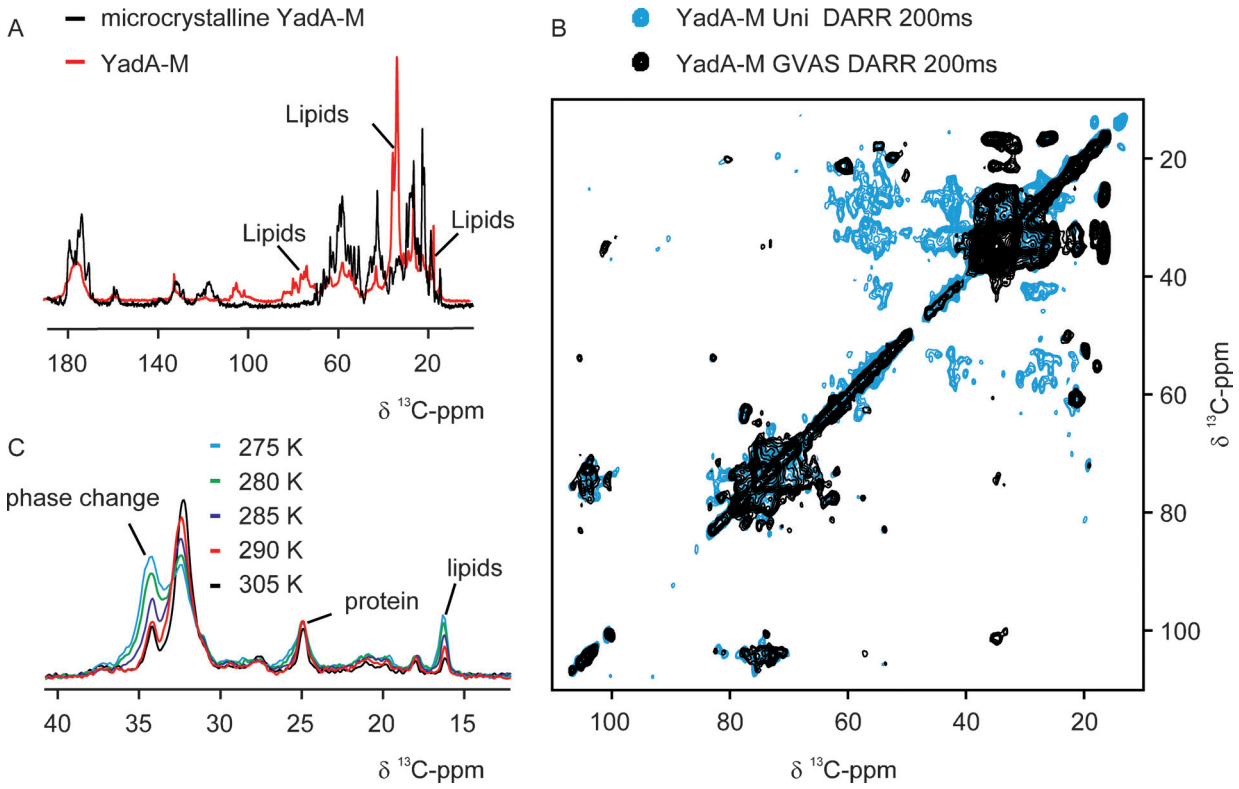

Figure 1. A) 1D spectra recorded for a uniformly labeled YadA-M microcrystalline sample (black) and uniformly labeled YadA-M in the OM (red). B) Overlay of 2D ^{13}C correlation spectra recorded with 200 ms dipolar assisted rotational resonance (DARR) mixing time for the GVAS- (black) and uniformly (cyan) labeled YadA-M samples in the OM. Lipid signals can be determined by spectral comparison. C) 1D observation of lipid phase transitions by recording ^{1}H-^{13}C CP-MAS spectra for the GVAS-labeled YadA-M samples at different temperatures.

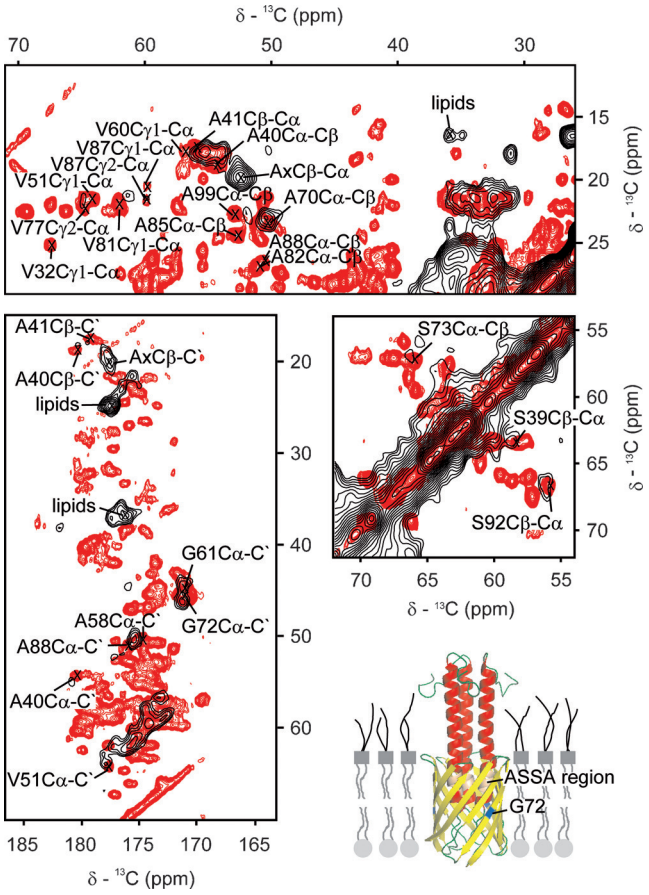

Figure 2. Assignment of chemical shifts from the GVAS-labeled YadA-M sample. The contour plots are taken from microcrystalline YadA-M (red) and a selectively GVAS-labeled preparation in the bacterial lipid bilayer. Both spectra were recorded with 25 ms DARR mixing at 275 K with similar acquisition parameters. At the bottom right is a cartoon model of YadA-M with secondary structural elements highlighted in different colors (α-helix red; β-sheet yellow; loop green). The highly conserved G72 and predicted hairpin-forming ASSA region are highlighted in gray and beige, respectively.

ssNMR data from selectively GVAS-labeled YadA-M were used to begin the chemical shift assignment procedure. Many cross-peaks in the GVAS-labeled sample overlapped well with the spectra for microcrystalline YadA-M (Figure 2), therefore, several assignments could be transferred directly. For instance, two sequential alanine residues in the α-helix (A40 and A41) show easily identifiable correlations in both the aliphatic and carbonyl regions, with no chemical-shift changes. On average, it appeared that residues that are not in direct contact with the lipid bilayer show higher intensities, for example, A70 shows a strong peak (Figure 2; top right panel). However, this could not be fully generalized since some of the residues facing the pore lumen (e.g., A82, A88) did not appear in the spectrum for the GVAS-labeled sample. Most valine residues face the lipid bilayer and either show weaker cross-peak intensities or do not appear, presumably owing to higher dynamics. Serine residues are mostly located at the more flexible β-turns and also do not show strong cross-peaks. The exception is the membrane-facing residue S73, which lies next to G72 in the second β-strand.[5] Our previous studies have shown S73 and G72 to be among the most rigid residues in microcrystalline YadA-M, and they show very strong sequential correlations even at short mixing times.[9a] In the OM YadA-M sample however, S73 shows far weaker correlations, most likely owing to the highly dynamic lipid environment, thus confirming that the dynamics of YadA-M are overall much higher in lipid bilayers.

The carbonyl region of the spectrum shows several cross-peaks from alanine and glycine residues, in particular from the biologically relevant and highly conserved residue G72, which is present in the second β-strand and faces the pore lumen. This residue plays a role in the autotransport process; replacing it with residues with larger side chains decreases the stability and autotransport efficiency of YadA.[5] As is evident from the spectral overlay, G72 does not show a change in chemical shift. This is also true for residues near the functionally important ASSA region since both A40 and A41 show almost unaltered chemical shifts in the aliphatic and carbonyl regions. This indicates that the non-helical chemical shifts of the ASSA region are inherent to the native structure and are not caused by any artefact during the crystallization of YadA-M.

We next recorded spectra on uniformly ^{13}C-labeled OM YadA-M samples at different mixing times to give intra-residue and interresidue correlations (Figure 3, bottom). Many residues that could not be assigned at shorter mixing times owing to peak overlap could be identified in spectra recorded with longer mixing times through sequential contacts, for example, the Y97 Cβ shows a sequential contact with N98 Cα in 200 ms DARR mixing to give an unambiguous assignment. Several residues were assigned in a similar manner, by using a strategy based on a pattern of backbone and side-chain sequential contacts.

Since glycine residues do not have side chains, they are only identifiable in a narrow strip of correlations between carbonyl groups and α-carbon atoms, thus making their assignment difficult. Still, most glycine residues in YadA-M (G52, G72, and G86) were unambiguously assigned through identification of sequential contacts in correlation spectra with extended mixing times. In addition to the sequential correlations, we tentatively assigned an interstrand contact between β-strands 3 and 4, that is, M96Cα–V87Cβ, which suggests the same β-strand registry as in microcrystalline YadA-M.

In the ssNMR structure for microcrystalline YadA-M, the β-barrel exterior is decorated with hydrophobic residues that form crystal contacts; these contacts can be directly observed in the ssNMR data (Figure 4). For instance, I71, I103, and some valine residues are among the hydrophobic residues involved in the formation of crystal contacts in microcrystalline YadA-M,[9b,14] and none of these residues show cross-peaks in the OM YadA-M sample.

While the NMR constraints observed in this study did not strictly confirm the trimeric structure, it is well established in previous studies and by the SDS PAGE gel shifts (Figure S2) that YadA in the OM forms a highly stable trimer.[9b] TALOS+[15] was used for secondary-structure prediction and to determine the random coil index (RCI) of YadA-M,

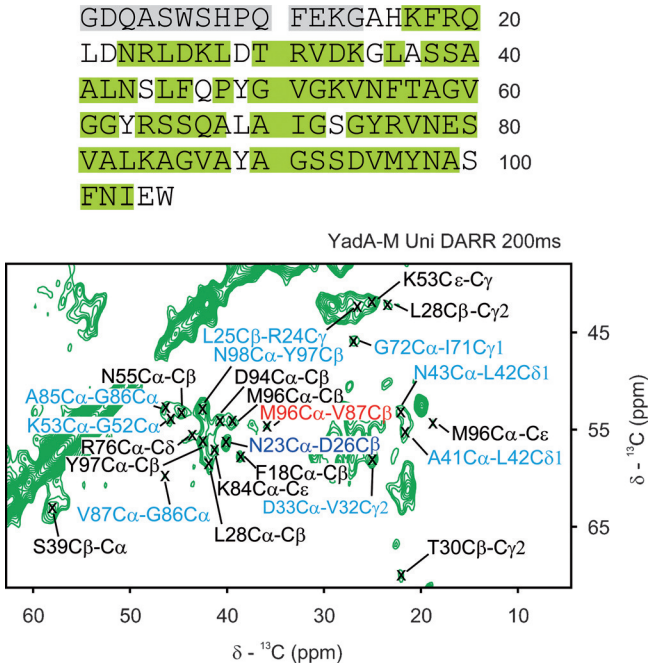

Figure 3. The top panel, with the sequence of YadA-M, shows those residues that were assigned in the bacterial OM samples highlighted in green (grey: strep-tag). Almost 75% of the observable residues have partial assignments. The spectrum on the bottom was recorded for uniformly labeled YadA-M with 200 ms DARR mixing with different types of correlations highlighted in black (intraresidue), cyan (sequential), dark blue (medium range), and red (long range).

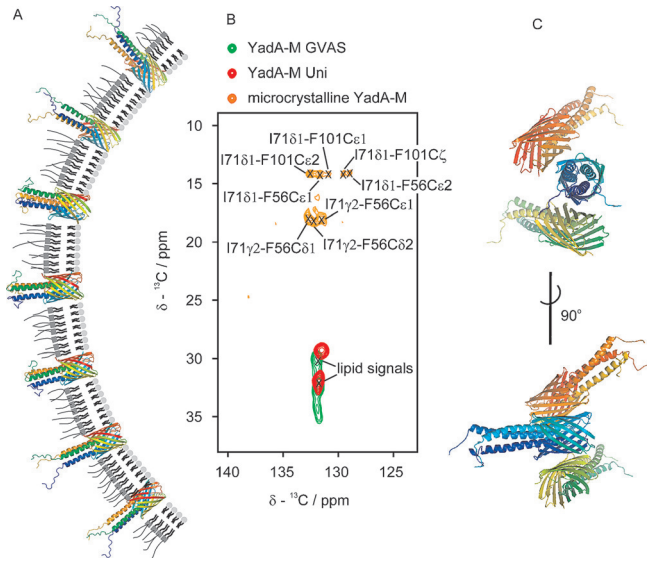

Figure 4. Crystal contacts missing in the MAS ssNMR data for the OM YadA-M sample. A) Representation of membrane-anchored YadA-M molecules in a bilayer. B) Assignments of crystal contacts from spectral overlay for YadA-M in the microcrystalline preparation and in the native membrane. C) Contact interface between YadA-M trimers as modeled by HADDOCK[13] on the basis of solid-state NMR crystal contacts.

although the TALOS predictions should be treated with caution owing to the absence of ^{15}N chemical shifts and missing ^{13}C backbone chemical shifts (Figure 5 and Figure S6). Higher dynamics are predicted in the linker region from residues L45 to V51 for the membrane-embedded preparation compared to the microcrystalline YadA-M sample (Figure 5). Moreover, the predicted order parameters for this region are low, which points towards higher flexibility of the linker region for the OM YadA-M sample (Figure S7). Furthermore, our previously reported finding that this stretch of residues (L45–V51) is part of the random-coil region that connects the N-terminal α-helix to the β-sheet is confirmed.[16]

We previously observed high flexibility for random-coil regions in microcrystalline YadA-M.[9b] Interestingly, higher RCI values for the linker region in YadA-M in the membrane suggest that Yad-M crystallization reduces the degree of freedom in this region. This is an intriguing result because this region lies inside the β-barrel and is not in direct contact with the dynamic lipid bilayer. Similarly, the ASSA region, which we have proposed to form an intermediate hairpin during autotransport in TAAs, also shows higher RCI values in the membrane-embedded YadA-M sample.

In summary, we show that our previously determined ssNMR structure of microcrystalline YadA-M remains essentially the same in the lipid bilayer, but that the dynamics of the lipid-exposed side-chains and loop regions of YadA-M are generally higher in the OM. NMR cross-peaks are generally less resolved in the OM owing to the inherently more heterogeneous environment, thus making spectral assignments more demanding. While proton-detected ssNMR can also be considered for high-resolution ssNMR studies, we did not use this strategy because backbone-amide exchange can be inefficient in membrane-embedded systems.[17] However, by using a combination of selectively and uniformly labeled samples, it is still possible to assign intra- and interresidue correlations, and to predict biologically relevant dynamics, which are often intimately linked to protein functionality, directly within the membrane. We believe this work demonstrates the feasibility of high-resolution structural and dynamic studies of proteins within their native environments at more physiological temperatures.

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Keywords: autotransport · membrane proteins · solid-state NMR · structural biology · *Yersinia* adhesin A

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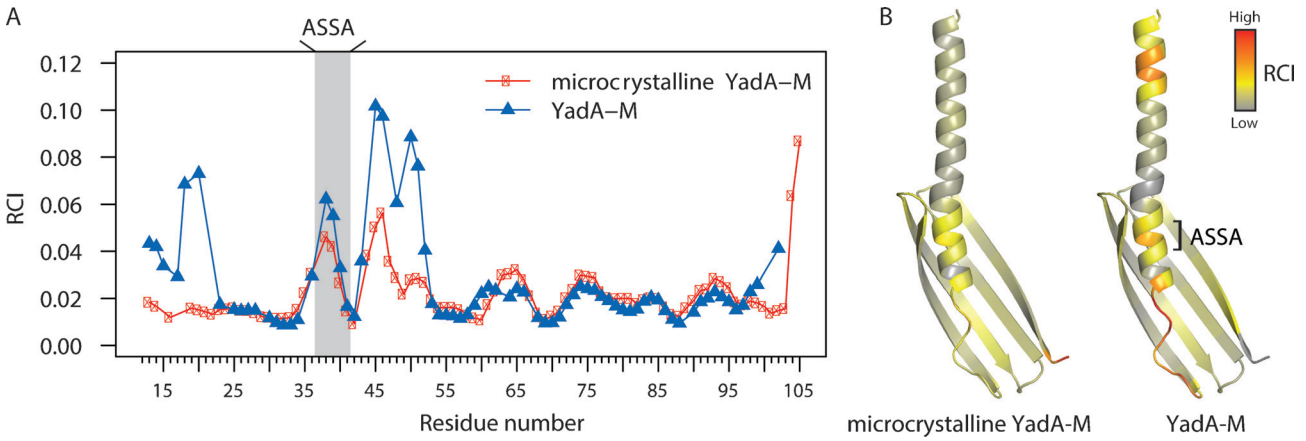

Figure 5. A) Comparison of TALOS+ predicted RCI values for YadA-M in microcrystals and membranes, with the OM Yad-M sample predicted to have several regions of increased flexibility. B) The YadA-M monomer structure highlighted according to the RCI values in microcrystalline and OM environments. The ASSA region is predicted to be especially flexible in both structures, but this is even more pronounced in the OM preparation.

[1] H. H. Niemann, W. D. Schubert, D. W. Heinz, *Microbes Infect.* **2004**, *6*, 101 – 112.
[2] D. Linke, T. Riess, I. B. Autenrieth, A. Lupas, V. A. Kempf, *Trends Microbiol.* **2006**, *14*, 264 – 270.
[3] J. C. Leo, I. Grin, D. Linke, *Philos. Trans. R. Soc. London Ser. B* **2012**, *367*, 1088 – 1101.
[4] C. J. Oomen, P. van Ulsen, P. van Gelder, M. Feijen, J. Tommassen, P. Gros, *EMBO J.* **2004**, *23*, 1257 – 1266.
[5] U. Grosskinsky, M. Schutz, M. Fritz, Y. Schmid, M. C. Lamparter, P. Szczesny, A. N. Lupas, I. B. Autenrieth, D. Linke, *J. Bacteriol.* **2007**, *189*, 9011 – 9019.
[6] a) K. Sackett, M. J. Nethercott, Z. Zheng, D. P. Weliky, *J. Mol. Biol.* **2014**, *426*, 1077 – 1094; b) S. O. Smith, *Annu. Rev. Biophys.* **2010**, *39*, 309 – 328; c) M. Weingarth, M. Baldus, *Acc. Chem. Res.* **2013**, *46*, 2037 – 2046.
[7] a) T. Sinnige, M. Weingarth, M. Renault, L. Baker, J. Tommassen, M. Baldus, *J. Mol. Biol.* **2014**, *426*, 2009 – 2021; b) M. Renault, M. P. Bos, J. Tommassen, M. Baldus, *J. Am. Chem. Soc.* **2011**, *133*, 4175 – 4177; c) M. T. Eddy, Y. Su, R. Silvers, L. Andreas, L. Clark, G. Wagner, G. Pintacuda, L. Emsley, R. G. Griffin, *J. Biomol. NMR* **2015**, *61*, 299 – 310.
[8] a) M. Renault, R. Tommassen-van Boxtel, M. P. Bos, J. A. Post, J. Tommassen, M. Baldus, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4863 – 4868; b) V. A. Higman, K. Varga, L. Aslimovska, P. J. Judge, L. J. Sperling, C. M. Rienstra, A. Watts, *Angew. Chem. Int. Ed.* **2011**, *50*, 8432 – 8435; *Angew. Chem.* **2011**, *123*, 8583 – 8586; c) M. Renault, S. Pawsey, M. P. Bos, E. J. Koers, D. Nand, R. Tommassen-van Boxtel, M. Rosay, J. Tommassen, W. E. Maas, M. Baldus, *Angew. Chem. Int. Ed.* **2012**, *51*, 2998 – 3001; *Angew. Chem.* **2012**, *124*, 3053 – 3056; d) V. S. Bajaj, M. L. Mak-Jurkauskas, M. Belenky, J. Herzfeld, R. G. Griffin, *J. Magn. Reson.* **2010**, *202*, 9 – 13.
[9] a) S. A. Shahid, S. Markovic, D. Linke, B. J. van Rossum, *Sci. Rep.* **2012**, *2*, 803; b) S. A. Shahid, B. Bardiaux, W. T. Franks, L. Krabben, M. Habeck, B. J. van Rossum, D. Linke, *Nat. Methods* **2012**, *9*, 1212 – 1217.
[10] a) H. X. Zhou, T. A. Cross, *Annu. Rev. Biophys.* **2013**, *42*, 361 – 392; b) Y. Miao, H. Qin, R. Fu, M. Sharma, T. V. Can, I. Hung, S. Luca, P. L. Gor'kov, W. W. Brey, T. A. Cross, *Angew. Chem. Int. Ed.* **2012**, *51*, 8383 – 8386; *Angew. Chem.* **2012**, *124*, 8508 – 8511.
[11] T. Jacso, W. T. Franks, H. Rose, U. Fink, J. Broecker, S. Keller, H. Oschkinat, B. Reif, *Angew. Chem. Int. Ed.* **2012**, *51*, 432 – 435; *Angew. Chem.* **2012**, *124*, 447 – 450.
[12] a) D. Chapman, *Q. Rev. Biophys.* **1975**, *8*, 185 – 235; b) P. Overath, M. Brenner, T. Gulik-Krzywicki, E. Shechter, L. Letellier, *Biochim. Biophys. Acta Biomembr.* **1975**, *389*, 358 – 369.
[13] a) C. Dominguez, R. Boelens, A. M. Bonvin, *J. Am. Chem. Soc.* **2003**, *125*, 1731 – 1737; b) S. J. de Vries, A. D. van Dijk, M. Krzeminski, M. van Dijk, A. Thureau, V. Hsu, T. Wassenaar, A. M. Bonvin, *Proteins Struct. Funct. Bioinf.* **2007**, *69*, 726 – 733.
[14] S. A. Shahid, Doctoral thesis thesis, Freie Universität Berlin (Berlin), **2012**.
[15] Y. Shen, F. Delaglio, G. Cornilescu, A. Bax, *J. Biomol. NMR* **2009**, *44*, 213 – 223.
[16] S. A. Shahid, M. Habeck, D. Linke, B. J. van Rossum, *GIT Lab. J.* **2013**, *17*, 14 – 16.
[17] a) R. Linser, M. Dasari, M. Hiller, V. Higman, U. Fink, J. M. Lopez del Amo, S. Markovic, L. Handel, B. Kessler, P. Schmieder, D. Oesterhelt, H. Oschkinat, B. Reif, *Angew. Chem. Int. Ed.* **2011**, *50*, 4508 – 4512; *Angew. Chem.* **2011**, *123*, 4601 – 4605; b) M. E. Ward, L. Shi, E. Lake, S. Krishnamurthy, H. Hutchins, L. S. Brown, V. Ladizhansky, *J. Am. Chem. Soc.* **2011**, *133*, 17434 – 17443.

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