Forschungsinstitut für Molekulare Pharmakologie Berlin

Structure determination of immobilized proteins by solid-state NMR spectroscopy

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List of papers

This thesis is based on the following publications:

I. Solid-state NMR structure determination of proteins by means of 3D ^{15}N-^{13}C-^{13}C dipolar correlation spectroscopy and chemical shift analysis.
F. Castellani**,** B. van Rossum, A. Diehl, K. Rehbein and H. Oschkinat, *Biochemistry* **2003**, 42,11476-83.

II. Assignment of amide proton signals by combined evaluation of HN, NN and HNCA MAS-NMR correlation spectra.
B. van Rossum, F. Castellani**,** J. Pauli, K. Rehbein, J. Hollander, H.J.M. de Groot and H. Oschkinat *J. Biomol. NMR* **2003**, 25, 217-223.

III. Structure of a protein determined by solid-state magic-angle spinning NMR spectroscopy.
F. Castellani, B. van Rossum, A. Diehl, M. Schubert, K. Rehbein and H. Oschkinat, *Nature* **2002**, 420, 98-102.

IV. Assignment of the nonexchanging protons of the α-spectrin SH3 domain by two- and three-dimensional ^{1}H-^{13}C solid-state magic-angle spinning NMR and comparison of solution and solid-state proton chemical shifts.
B. van Rossum, F. Castellani, K. Rehbein, J. Pauli and H. Oschkinat *ChemBioChem* **2001,** 2, 906-914.

Further publications:

V. ^{1}H detection in MAS solid-state NMR spectroscopy of biomacromolecules employing pulsed field gradients for residual solvent suppression.
V. Chevelkov, B. J. van Rossum, F. Castellani**,** K. Rehbein, A. Diehl, M. Hohwy, S. Steuernagel, F. Engelke, H. Oschkinat and B. Reif *JACS* **2003**, 125, 7788-7789.

VI. Characterization of ^{1}H-^{1}H distances in a uniformly ^{2}H,^{15}N-labeled SH3 domain by MAS solid-state NMR spectroscopy.
B. Reif, B. van Rossum, F. Castellani**,** K. Rehbein, A. Diehl and H. Oschkinat, *JACS* **2003**, 125, 1488-1489.

VII. Partial orientation of cytochrome *c* in a lyotropic liquid crystal: residual H-H dipolar coupling.
I. Bertini, F. Castellani, C. Luchinat, G. Martini, G. Parigi, S. Ristori *J. Phys. Chem. B* **2000**, 104 (45), 10653-10658.

Abbreviations

1D, 2D, 3D	one dimensional, two dimensional, three dimensional
Å	Ångstrøm (1 Å = 0.1 nm)
CNS	Crystallography and NMR System
CP	cross polarization
CSA	chemical shift anisotropy
CW	continuous wave
FID	free induction decay
FSLG	frequency- and phase-switched Lee-Goldburg
γ	giromagnetic ratio
HSQC	heteronuclear single quantum coherence
kDa	kilo Dalton
LG	Lee-Goldburg
MAS	magic-angle spinning
NMR	nuclear magnetic resonance
PDSD	proton-driven spin diffusion
PMLG	phase-modulated Lee-Goldburg
ppm	part per million
rf	radio frequency
RFDR	radio-frequency driven dipolar recoupling
rmsd	root-mean square deviation
SH3	Src-homology 3
2-SH3	SH3 sample obtained by growth on [2-^{13}C] glycerol
1,3-SH3	SH3 sample obtained by growth on [1,3-^{13}C] glycerol
SW	spectral window
T_1	longitudinal relaxation time
T_2	transversal relaxation time
TPPM	two-pulse phase modulation

Summary

Recently, rapid progress has been made in the field of biological solid-state MAS NMR with regards to sensitivity, resolution and full resonance assignment. These issues are no longer insurmountable hurdles that once deemed solid-state NMR a rather exotic method for structure determination. This thesis demonstrates the forthcoming role of solid-state MAS NMR as a mainstream technique for structural investigation of proteins, as a method complementary to solution NMR and single-crystal diffraction. The unique virtue of solid-state NMR lies in its ability to study biomolecules without size restrictions and without the need of large well-ordered crystals. Hence, solid-state NMR has a strong potential to enable the structural investigation of several classes of biological systems, such as amyloid fibrils and membrane proteins, which are not easily accessible by the above-mentioned methods.

In this thesis, a simple methodology is presented for determining the structure of proteins by solid-state MAS NMR. As a key step, a large number of carbon-carbon distance restraints in the range of 2–7 Å is collected, from a small number of samples and with minimal experimental effort. The method is demonstrated with the calculation of the β-sandwich fold of the 62-residue SH3 domain of α-spectrin. This structure represents the first protein structure determined by solid-state MAS NMR. The set of constraints may be supplemented by ^{1}H-^{1}H restraints obtained from proton-proton correlation spectroscopy using samples deuterated at non-exchangeable sites, or from ^{13}C-^{15}N restraints from C–N dipolar correlation spectroscopy using doubly labelled samples or selective recoupling in combination with multiply labelled samples. In the foreseeable future, techniques will be developed to include ^{1}H-^{13}C and/or ^{1}H-^{15}N restraints.

The initial step in determining a structure by NMR is the resonance assignment. Chapter 2 describes side-chain and sequential assignment strategies applied to the α-spectrin SH3 domain, that is used as an example. An almost complete ^{13}C and ^{15}N resonance assignment of a uniformly ^{13}C- and ^{15}N-labelled SH3 sample was achieved previously (Pauli *et al.*, 2001) by multidimensional homonuclear and heteronuclear correlation spectroscopy. Based on the ^{13}C and ^{15}N assignment, we arrived at an almost complete solid-state NMR assignment of proton signals by means of heteronuclear dipolar correlation spectroscopy, using indirect ^{1}H detection. The resonances of non-exchangeable protons were assigned by 3D ^{1}H-^{13}C-^{13}C correlation spectroscopy. A novel 3D (^{1}H-^{15}N-^{13}C) heteronuclear correlation experiment was used for the amide proton resonance assignment.

The main focus of this thesis is the development of a strategy for the collection of a large number of distance restraints for structure determination. For this purpose, we have devised a methodology that allowed the measurement of multiple ^{13}C-^{13}C long-range distances by combining broad-band recoupling methods with dilution of ^{13}C spins. The general outline of the method is presented in Chapter 3.

The biosynthetically site-directed ^{13}C-enriched samples needed for the measurement of long-range constraints were obtained by growing bacteria on [2-^{13}C] glycerol and [1,3-^{13}C] glycerol. Chapter 4 describes how we determined the labelling pattern of the two biosynthetically site-directed ^{13}C-enriched samples, using several solution NMR experiments.

The spectroscopic technique we chose for the collection of structural-defining ^{13}C-^{13}C distances is the proton-driven spin diffusion (PDSD) recoupling method. In Chapters 5 and 6, it is described how two- and three-dimensional dipolar correlation spectroscopy was applied to the selectively-enriched α-spectrin SH3 samples. A structure was derived from solid-state data only, with a backbone root-mean square-deviation of 0.7 Å.

In Chapter 7, the structural information contained in the chemical-shift was exploited by analysing the differences in solution and solid-state NMR chemical-shifts of the SH3 domain. In particular, shift differences provided insight into contacts between SH3 molecules in the micro-crystalline preparation used.

The ^{13}C-^{13}C long-range correlations obtained with the PDSD recoupling method were converted into distance restraints using an empirical approach. Nitrogen spins form a less dense network of spins than carbon nuclei. In Chapter 8, we investigated in more detail the mechanism of magnetization transfer in ^{15}N-^{15}N proton-driven spin diffusion experiments

between ^{15}N spins. A simple method was derived to extract distance information from build-up curves of cross-peak intensities in ^{15}N-^{15}N PDSD spectra.

The study of bacterial membrane proteins within structural genomics projects is an obvious next application of the novel technique described herein, together with the study of receptor-bound agonists and antagonists.

Zusammenfassung

Auf dem Gebiet der biologischen Festkörper-MAS-NMR wurden in der letzten Zeit enorme Fortschritte hinsichtlich der Empfindlichkeit, Auflösung und der vollständigen Zuordnung aller Signale erzielt. Damit wird es nun möglich, die Festkörper-NMR auch für die Strukturbestimmung von Proteinen zu nutzen. Die vorliegende Arbeit zeigt die zukünftige Rolle der Festkörper-MAS-NMR als eine weitere wesentliche Technik für die Strukturuntersuchungen von Proteinen, neben der Flüssigkeits-NMR und der Kristallographie. Der besondere Wert der Festkörper-NMR liegt in der Möglichkeit der Strukturbestimmung von beliebig großen Biomolekülen, von denen keine gut brechende Kristalle erzeugbar sind. Die Festkörper-NMR ermöglicht daher die strukturelle Untersuchung verschiedener Klassen biologischer Systeme, wie zum Beispiel Amyloidfibrillen und Membranproteine, die mit anderen Methoden der Strukturbestimmung nicht oder nur schwer zugänglich sind.

In der vorliegenden Arbeit wird eine einfache Methode vorgestellt, um die Strukturbestimmung von Proteinen mittels Festkörper-NMR zu ermöglichen. Das Kernstück dieser Methode ist die Messung einer großen Anzahl von Kohlenstoff-Kohlenstoff-Abständen im Bereich von 2-7 Å, wobei sowohl die Probenanzahl, als auch der experimentelle Aufwand gering gehalten werden kann. Die Methode wurde zur Berechnung der β-Sandwich-Struktur der SH3-Domäne aus α-Spectrin (62 Aminosäuren) eingesetzt und evaluiert. Dies ist die erste Strukturbestimmung eines Proteins, welche mit der Festkörper-MAS-NMR durchgeführt wurde. Weitere Strukturparameter können zukünftig durch Extraktion von Abständen aus ^{1}H-^{1}H- oder ^{13}C-^{15}N-Korrelationen erhalten werden. ^{1}H-^{1}H-Korrelationen werden an nichtaustauschbaren Protonen deuterierter Proben mittels Proton-Proton

Korrelationsspektroskopie erzeugt. Für ^{13}C-^{15}N-Korrelationen wiederum nutzt man doppelt markierte Proben und C-N-dipolare Spektroskopie oder mehrfach markierte Proben und selektive Kopplungsspektroskopie. In absehbarer Zeit werden Techniken entwickelt werden, um ^{1}H-^{13}C und/oder ^{1}H-^{15}N Bedingungen einzubeziehen.

Der erste Schritt bei der Bestimmung einer NMR-Struktur ist stets die Resonanzzuordnung. In Kapitel 2 werden die Seitenketten- und Sequenzzuordnungsstrategien beschrieben, die auf α-Spectrin-SH3 als Präzedenzfall angewandt wurden. Eine fast vollständige ^{13}C- und ^{15}N-Resonanzzuordnung durch mehrdimensionale homo- und heteronukleare Korrelationsspektroskopie wurde für die uniform ^{13}C- und ^{15}N-markierte-SH3 Probe bereits im Vorfeld erarbeitet (Pauli *et al.*, 2001). Darauf basierend wurde eine fast vollständige Festkörper-NMR-Zuordnung von Protonensignalen mittels heteronuklearer Korrelationsspektroskopie durch indirekte ^{1}H-Messung erreicht. Die Resonanzen nicht-austauschbarer Protonen wurden durch 3D-^{1}H-^{13}C-^{13}C-Korrelationspektroskopie zugeordnet. Ein neues 3D-(^{1}H-^{15}N-^{13}C) heteronukleares Korrelationexperiment wurde für die Amid-Protonen-Resonanzzuordnung entwickelt.

Das Hauptziel dieser Arbeit lag in der Entwicklung einer Strategie zur Messung einer großen Anzahl von Abständen, die für die Strukturrechnung unabdingbar sind. Dafür wurde eine Methode etabliert, die die Messung von vielen Weitbereichs-^{13}C-^{13}C-Abständen erlaubt, indem eine breitbandige "recoupling"-Methode mit selektiven ^{13}C-Markierungstrategien kombiniert wird (Kapitel 3).

Die Bestimmung von Abständen über weite Bereiche erfordert selektiv-markierte ^{13}C-Proteinproben, die biosynthetisch erzeugt werden können, indem Bakterien entweder auf [2-^{13}C]-Glycerol oder auf [1,3-^{13}C]-Glycerol als einziger C-Quelle angezogen werden. Kapitel 4 beschreibt, wie das Markierungsmuster der zwei biosynthetisch spezifisch-markierten ^{13}C-Proben mittels Flüssigkeits-NMR bestimmt wurde.

Als spektroskopische Technik zur Messung von Struktur-bestimmenden ^{13}C-^{13}C-Korrelationen wurde die "proton-driven spin diffusion" (PDSD) Korrelationsspektroskopie gewählt. In den Kapiteln 5 und 6 wird beschrieben, wie diese Technik in 2D- und 3D-Experimenten auf die selektiv-markierten SH3-Proben angewandt wurden. Mit den extrahierter Abstandsinformationen konnte eine lediglich auf Festkörper-NMR-Daten beruhende Struktur mit einem RMSD-Wert von 0.7 Å berechnet werden.

In Kapitel 7 wurden die Unterschiede zwischen den Flüssigkeits- und Festkörper-NMR chemischen Verschiebungen der SH3-Domäne genutzt, um Strukturinformationen zu erhalten. Diese Verschiebungsunterschiede ermöglichen Einblicke in die intermolekularen Kontakte zwischen SH3-Domänen in der eingesetzten mikrokristallinen Präparation.

Aus den Weitbereichs-^{13}C-^{13}C-Korrelationen, die mittels PDSD-Technik erhalten wurden, konnten über einen empirischen Ansatz Abstände bestimmt werden. Stickstoffkerne bilden ein weniger dichtes Netzwerk als die Kohlenstoffkerne. In Kapitel 8 wird der Übergang der Magnetisierung zwischen ^{15}N-Kernen in ^{15}N-^{15}N-PDSD-Experimenten detaillierter untersucht. Eine einfache Methode zur Erzeugung von Abstandsinformationen aus ansteigenden Kurven von Kreuzsignalintensitäten in ^{15}N-^{15}N-PDSD-Spektren wurde entwickelt.

Die in der vorliegenden Arbeit neu eingeführte Methode wird mit Sicherheit bei den strukturellen Untersuchungen von bakteriellen Membranproteinen im Rahmen von Genomprojekten und beim Studium von Rezeptor-gebundenen Agonisten und Antagonisten Anwendung finden.

Curriculum vitae

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Oct 1993 – Apr 1999 Laurea in Chemistry (master thesis), Department of Chemistry, University of Florence (Italy).

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Supervisor: Professor Ivano Bertini

Classification: 110 /110 *cum laude*

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