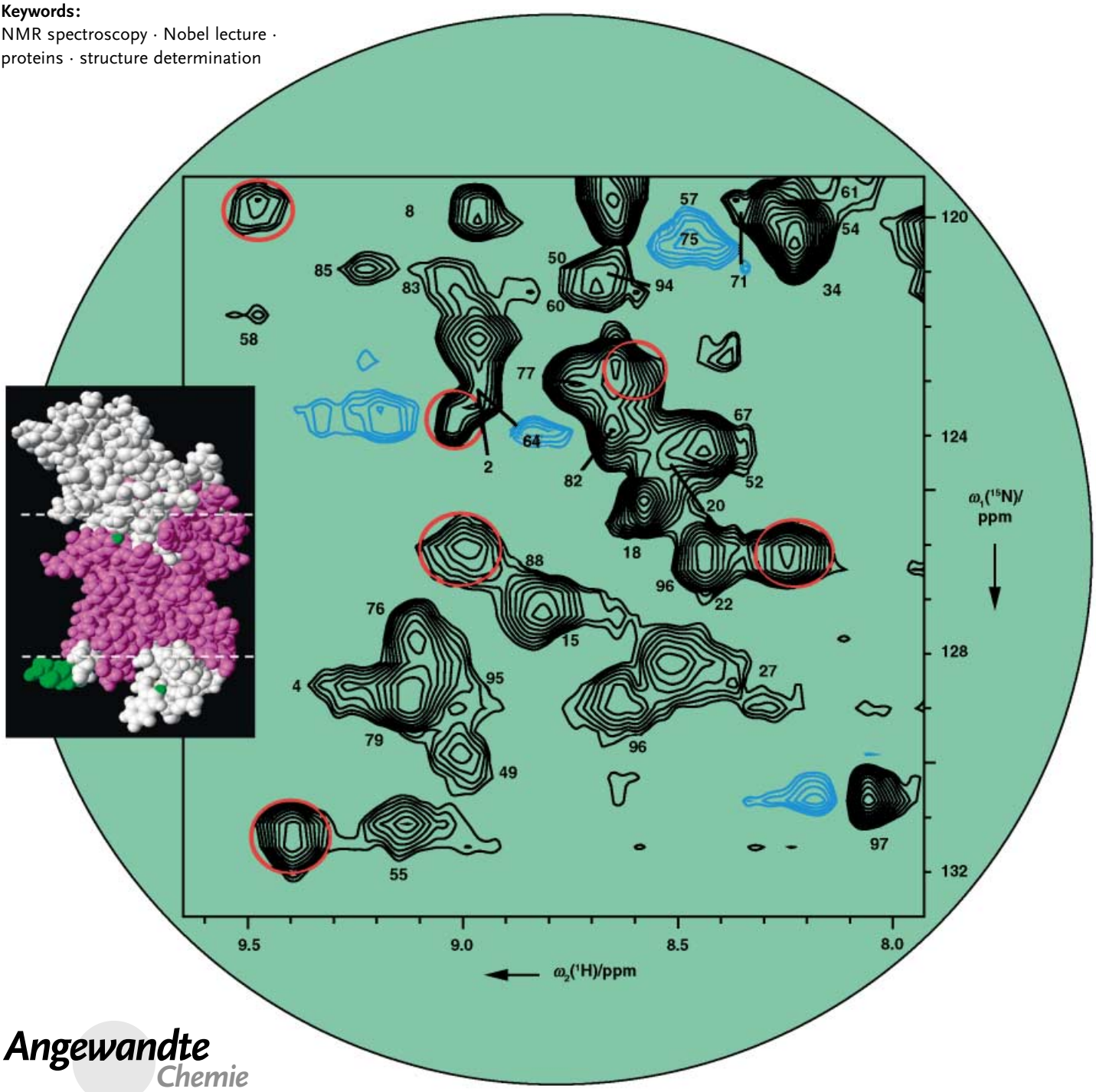
NMR of Biomacromolecules

NMR Studies of Structure and Function of Biological Macromolecules (Nobel Lecture)**

*Kurt Wüthrich**

Keywords:
NMR spectroscopy · Nobel lecture · proteins · structure determination



Angewandte Chemie

  DOI: 10.1002/anie.200300595

Angewandte Chemie

From the Contents

Biographical Note

I was born in Aarberg, Switzerland, on October 4, 1938, and during my childhood I lived in the small town of Lyss in the Berner Seeland. At the time this was a rural area of farmland, forests, and rivers. The roots of the Wüthrich family are in an even more rural, mountainous area, the farming village of Trub in the Emmental near Bern. My mother's family owned the restaurant "Bären" and a bakery in Lyss. My grandfather, Otto Kuchen, enjoyed fishing and hunting, and his jugged hare dish was a widely known fall season delicacy at the "Bären". My interests during childhood were largely influenced by our living in an old farmhouse, where my second grandfather, Jakob Wüthrich, had been a farmer. Although my father, Herrmann Wüthrich, took up an occupation as an accountant, he remained very much attached to his upbringings and our family produced a wide range of farming goods. My mother, Gertrud Wüthrich-Kuchen was the true center of our family life. In addition to raising me and my two younger sisters, Elisabeth and Ruth, she did marvelous things in the kitchen, tended our big garden, raised fowl, and was involved in various activities in the community.

My intense contacts with the rural environment of plants and animals awakened my interest in natural science at an early age. In particular, I acquired a thorough knowledge of the behavior of all sorts of water animals, mostly through observations made while enjoying all aspects of work and fun with a private trout river. On rare occasions I still enjoy fishing trips, and am a member of the Mercury Bay Game Fishing Club in Whitianga, New Zealand, which lists Ernest Hemingway and Zane Grey among its all-time membership. With regard to my professional life, I had set my mind on becoming a forest engineer. Although I subsequently changed my mind in this regard, I still enjoy tending the family forest, which now

With my parents, Herrmann and Gertrud Wüthrich-Kuchen, and my two sisters, Elisabeth and Ruth, in the garden of our home in Lyss, Switzerland, 1944.

At the Mercury Bay Game Fishing Club in Whitianga, New Zealand, 1987.

[*] Prof. Dr. K. Wüthrich
Institut für Molekularbiologie
Eidgenössische Technische Hochschule Zürich
8093 Zürich (Switzerland)
Fax: (+41) 1 633 11 51
E-mail: kurt.wuthrich@mol.biol.ethz.ch
and
The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)

 DOI: 10.1002/anie.200300595

contains trees that were planted by three generations of our family, starting with my grandfather.

My formal training toward an academic profession started in 1952, when I transferred from the village school in Lyss to the Gymnasium in the nearby "bilingue" city of Biel/Bienne. During the Gymnasium years my interests widened beyond forestry and fishing. We had the good fortune that our science and language teachers were either former university professors, who had left their academic positions elsewhere in Europe during the Second World War and found a haven in Biel, or followed the then common practice of using a teaching assignment at Gymnasium level as a stepping-stone for an academic career. At age 14 to 18 we were a group of seven students specializing in natural sciences who were thus trained in mathematics and physics at university level, and I happily accepted the challenge. According to my mother, it was during those years that I got used to working through the nights. Another focus was the French language, French literature, and French theatre and movies, which was largely motivated by the fact that the composition of our class as well as our teachers represented the bilingual character of Biel/Bienne. The Gymnasium Biel was informally attached to the Swiss Federal School of Sports and Gymnastics in nearby Magglingen, and thus my interest in competitive sports was awakened. These three areas all play an important role in my life up to the present days. Physics and mathematics are key activities in my professional life, professional visits in Paris and "les provinces" are combined with the sampling of French food, wine, and culture, and I not only obtained the "Eidgenössisches Turn- und Sportlehrerdiplom" (Swiss Federal Gymnastics and Sports Instructor Degree) as one of my University degrees, but also played in a competitive soccer league well beyond the age of 50.

By now I can look back on 40 years of intense involvement with techniques referred to as magnetic resonance spectroscopy. At the outset in 1962 and throughout my graduate studies there was electron paramagnetic resonance (EPR spectroscopy). EPR was complemented during my postdoctoral training from 1965 to 1967 by nuclear magnetic resonance (NMR) spectroscopy applied to chemical physics projects, and since the fall of 1967 I have used NMR for studies of biological macromolecules. From there it was a sinuous avenue that led by 1984 to the NMR method for protein structure determination in solution. Our results were occasionally met with doubts and disbelief, so that considerable moral strength and perseverance were at times called for.

During my student years from 1957 to 1962, NMR spectroscopy was just being introduced as an analytical tool in chemistry, molecular biology was not yet established as an independent discipline, and the initial three-dimensional protein crystal structures at atomic resolution were just emerging. My education at the University of Bern could thus not possibly cover the areas of our current research. The faculty and the student classes in Bern were small in numbers, with three physics students and seven chemistry students starting in 1957. From my curriculum in chemistry, physics, and mathematics, I best remember intense work in linear algebra, classical mechanics, chemical thermodynamics, physical chemistry of synthetic polymers, and preparative biochemistry of proteins and nucleic acids. This combination turned out to be an excellent foundation for my later scientific activities. The last two years of formal education, from 1962 to 1964, were spent at the University of Basel, majoring in sports and obtaining a Ph.D. in chemistry. Studying sports included about 25 weekly hours of intense physical exercise as well as premedical courses in human anatomy and physiology. Combined with experience gained from observations made on myself in the pursuit of competitive sports, this provided an additional dimension to my education. The subject of my Ph.D. thesis in inorganic chemistry with Professor Silvio Fallab was the catalytic activity of copper compounds in autoxidation reactions, and for this project the availability of a state-of-the-art EPR spectrometer in the Physics Institute was a great opportunity.

As a postdoctoral fellow in Berkeley, 1966.

Studying natural sciences has always been a lot of fun for me, but nonetheless my mind was quite solidly set on a career as a high-school teacher with a heavy involvement in sports. In parallel to my studies in natural sciences, I extensively yielded to what I thought to be my vocation. Thus, during the years 1957–1962, I spent part of each winter as a ski instructor in Swiss mountain resorts. From 1959 to 1965, I had part-time jobs in high schools, first teaching physics at the Kantonsschule Solothurn, then chemistry at the Gymnasium Biel, and finally gymnastics at the Mädchengymnasium in Basel. These teaching assignments also had an important impact on my personal life: In 1961, while on my job as a ski instructor in the resort town of Saanenmöser in the Berner Oberland, I met my wife, Marianne Briner, who at the time was an elementary school teacher. We were married in 1963, and Marianne then joined me in studying sports at the University of Basel, graduating with the "Eidgenössisches Turn- und Sportlehrerdiplom" and specializing in modern dance. After the graduate student and postdoctoral years we started

With my wife Marianne, son Bernhard, and daughter Karin in front of our apartment building in Greifensee, Switzerland, 1972.

Professor Robert E. Connick in my office at the ETH Zürich, 1981.

With, from left to right, Karin, Marianne, and Bernhard in the desert near Tucson, AZ, USA, 1984.

a family, with our son Bernhard Andrew being born in 1968 in Berkeley Heights, NJ, USA, and our daughter Karin Lynn joining us in 1970 in Greifensee near Zürich, Switzerland.

After finishing my graduate studies I spent another year in Basel concentrating on EPR studies of metal complexes in solution. In the spring of 1965 we moved to the USA, where I joined Professor Robert E. Connick at the University of California, Berkeley, for postdoctoral training. We used NMR spin relaxation measurements of ^{17}O, ^{2}H, and ^{1}H in addition to EPR for studies of the hydration of metal ions and metal complexes. The Berkeley period was devoted to intensive work on the theory of nuclear spin relaxation, group theory, and quantum mechanics, which was motivated by Bob Connick's weekly group seminar, a graduate course on Group Theory and Quantum Mechanics by Michael Tinkham, and an intense collaboration with another Swiss postdoc, Alex von Zelewsky, who soon thereafter accepted the chair of inorganic chemistry at the University of Fribourg in Switzerland. Over the years, Marianne and I returned at regular intervals to Berkeley to renew the friendships of the 1960s and revive fond memories.

In October 1967 I joined the Biophysics Department of Dr. Robert G. Shulman at Bell Telephone Laboratories in Murray Hill, New Jersey. I was given responsibility for the maintenance of one of the first superconducting high-resolution NMR spectrometers, which operated at a proton resonance frequency of 220 MHz, and I was otherwise free to use this instrument for research on protein structure and function. Due to my background, my interest was focused on metal centers rather than on polypeptide chains, and all my initial projects in high-resolution NMR had to do with hemoproteins. Using blood sampled from my arm in the first aid station, a Japanese colleague at Bell Laboratories, Dr. Tetsuo Yamane, prepared "hemoglobin (KW)", and within a few months we found entirely new avenues of deriving information on structure–function correlations from the NMR spectra of hemoglobin and other hemoproteins. These projects were a lucky choice: with the limited sensitivity and spectral resolution of the instrumentation available in 1968, the special spectral properties of hemoproteins were a great asset for successful NMR applications. Many years later, the unique NMR spectral features that enabled the early work with these metalloproteins had an important role in various aspects of the development of the NMR method for three-dimensional protein structure determination.

In October 1969 I returned to Switzerland to join the ETH Zürich. From the start I was equally well equipped with NMR and EPR instrumentation as previously at Bell Telephone Laboratories, and during the following 32 years the ETH provided us in regular

Visiting the University of California, Berkeley, 1997. Dudley Herschbach happened to visit on the same day. Lunch at "Chez Panisse" was running late, so our host, Alex Pines, carried the desserts along to the seminar room.

intervals with the most advanced NMR equipment. Until 1975 I was working with a small group of students, a chemical engineer, Rudolf Baumann, who has stayed with me throughout all these years, and a postdoctoral associate with a physics Ph.D. in solid state EPR, Dr. Regula Keller, who pursued highly successful research with hemoproteins from 1970 to 1982. In 1973, Gerhard Wagner decided to do his graduate work with me. Gerhard then stayed with the group until 1987, pursuing a classical European academic career with Habilitation before settling as a Professor at Harvard Medical School. Being able to keep outstanding junior scientists as research associates over extended periods of time was a special privilege enjoyed by senior faculty in the traditional European system, and the continued presence of Rudolf, Regula, and Gerhard during most of my initial 15 years in Zürich was a key factor for success with our research program.

In Zürich, we continued research on hemoproteins with the use of NMR and EPR spectroscopy, where the biochemical work was mostly done by groups outside of the ETH who joined us for collaborative projects, and the spectroscopic work was done by Regula Keller, myself, and a succession of graduate students. In addition, we started a program of systematic studies on the application of NMR techniques to polypeptides and small proteins.

Spirits were kept high by successful studies of cyclic peptides in collaboration with the Head of the Institute of Molecular Biology and Biophysics, Prof. Robert Schwyzer, the observation of unexpectedly well-resolved and long-lived NMR lines of amide protons in the small protein basic pancreatic trypsin inhibitor (BPTI), and the discovery of ring flips in BPTI. On the main line of research, which was to develop a method for protein structure determination in solution, there was only little progress. In 1975, in an attempt to survey the state of the field of NMR spectroscopy with biological macromolecules, I wrote the monograph *NMR in Biological Research: Peptides and Proteins*. There were two principal conclusions from this venture that would greatly affect the continuation of our work plan. First, I fully realized that we had been extremely fortunate in choosing hemoproteins as a focus for our early NMR efforts. Second, it became clear that attempts of the early 1970s to derive de novo three-dimensional protein structures from conformation-dependent proton chemical shifts was not a promising approach, independent of whether these shifts were caused by intrinsic or extrinsic diamagnetic or paramagnetic probes. We thus had to look for novel avenues for NMR structure determination, where hemoproteins with their unique NMR spectral properties could be an ideal testing ground for new ideas.

Shortly after I had learned my lessons from writing the 1976 monograph, the conditions under which I could pursue my work evolved in quite important ways. After working for more than 5 years with a small group of students and research associates from the environs of Zürich, and being able to spend long hours of my own time at the bench and on the NMR spectrometers, I found myself suddenly surrounded by more than 20 postdoctoral fellows and students from all over the world. At around the same time, I also started to travel quite extensively in all parts of the world, with a first visit to India at the end of 1974, and a first round-the-world trip including stops in the USA and in Japan in the fall of 1975. The visits to India and Japan resulted in new, lasting friendships with local colleagues, and also in attracting a number of most talented postdoctoral fellows to Zürich. Ever since, professional travel has become an important part of my activities. Over the years this also included visiting faculty appointments at the University of California, Berkeley, Cornell University in Ithaca, NY, Johns Hopkins University in Baltimore, MD, the California Institute of Technology in Pasadena, CA, the Scripps Research Institute in La Jolla, CA, RIKEN in Tokyo, Japan, and the University of Edinburgh, UK. Spending part of my time in these places of highest standards added greatly to my quality of life as well as to the progress of our research.

The international aspect of my activities received a special boost in 1975, when—out of the blue—I was elected to membership in the Council of the International Union of Pure and Applied Biophysics (IUPAB). There was little work involved in this assignment, but in 1978 my IUPAB affiliation changed to being its Secretary General, and with this I also became a member of the General Committee of the International Council of Scientific Unions (ICSU) and of the ICSU Standing Committee on the Free Circulation of Scientists. During the six-year term as Secretary General the demands on my time were thus quite heavy. Fortunately, Marianne agreed to run the IUPAB office. This made things easier, since she would travel with me and we dealt with the IUPAB business in makeshift offices temporarily installed in hotels all around the world. The sunny side was that I got to know many prominent scientists, whose names I had previously mostly known from textbooks. For example, structural biology was represented in the IUPAB Council from 1978–1981 by Britton Chance, Henryk Eisenberg, David Phillips, Frederic Richards, and Akiyoshi Wada, a true center of excellence! In the business meetings as well as in the social gatherings, we spent much of our time discussing the latest research advances long before they appeared in print. There was a particularly close collaboration with the IUPAB Presidents during my tenure as Secretary General, Professor Setsuro Ebashi and Professor Richard Keynes. Richard Keynes is a great-grandson of Charles Darwin. During IUPAB-related joint travel in Europe and the Far East in 1982/83, I listened to a more and more enjoyable but seemingly endless series of presentations of his Darwin Lecture commemorating the 100th anniversary of Darwin's death; in return, Richard lived through a heavy dose of biomolecular NMR spectroscopy.

Through my association with ICSU and IUPAB, I also became involved in entirely novel business. Most notable in hindsight were negotiations during the period 1980–1983 about joint adherence of China and Taiwan in international science organizations. We eventually defined terms and conditions for adherence to IUPAB of both "The Biophysical Society of China located in Beijing, China" and "The Chinese Biophysical Society located in Taipei, China". This involved extensive, highly formal correspondence, as well as visits and personal negotiations with Government and Academy officials in both countries. I also participated in IUPAB and ICSU programs of support for scientists in developing countries, and I organized summer schools and symposia in Africa, the Far East, and Latin America. This all greatly influenced my outlook on the world. Although each year the IUPAB-related activities and my research-related travel kept me out of my laboratory for several months, the effect on our research was overall highly beneficial. As a bonus, I

The President and the Secretary General of the IUPAB with their wives during a diplomatic mission in China, 1983. President Professor Richard Keynes and Ann Keynes are on the left and right, respectively.

At dinner during an ICSU General Committee meeting in Munich, Germany, 1985. To my left is Professor Jorge Allende from Chile, to my right is Professor Raymondo Villegas from Venezuela.

Addressing the audience during the opening session of an IUPAB/ICSU/UNESCO-sponsored "Winter School on Magnetic Resonance in Biology and Medicine" in Cairo, Egypt, 1986. To my left is Professor I. El Gohari, who has over the years been the Egyptian delegate to numerous IUPAB functions.

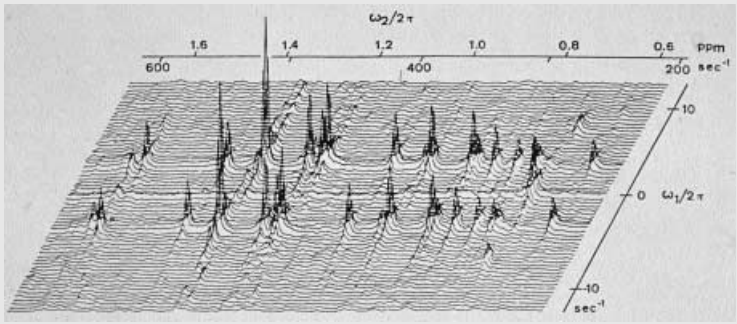
With Professor Chen-Lu Tsou, enjoying the beautiful scenery of Wuxi, China, during an IUPAB/ICSU-sponsored Summer School on Biophysics, 1992.

A dinner party during a visit with Professor Setsuro Ebashi (second from the right) in Okasaki, Japan, 1996. Mrs. Ebashi is next to Marianne, and Kuniaki Nagayama, who started 2D NMR with proteins as a postdoctoral fellow with Richard Ernst and myself in the late 1970s, is on the left wing.

gained experience in directing a research group at a distance, and my junior associates could test their own initiatives during my absences.

With all the new talent assembled in my group by 1976, we started to develop new NMR experiments and novel algorithms for the structural interpretation of NMR data, which eventually resulted in the NMR method for protein structure determination. This included the identification of the nuclear Overhauser effect (NOE) as a NMR parameter that can be related in an unambiguous way to three-dimensional macromolecular structures. We made use of the outstanding resolution of parts of the hemoprotein NMR spectra for calibrating NOE distance measurements with the then-available one-dimensional (1D) NMR techniques. In addition to Regula Keller, Sidney Gordon, a sabbatical visitor, made key contributions with the introduction of the 1D transient NOE experiment. Subsequently, the NOE had a key role in the approach used for obtaining sequence-specific assignments of the many hundred to several thousand NMR lines in a protein. The sequential assignment strategy was initially implemented by Gerhard Wagner and a diploma student, Andreas Dubs, using 1D NOE and spin-decoupling experiments. In parallel with the 1D NMR investigations on NOEs and NMR assignment in proteins, the development of two-dimensional (2D) NMR techniques for macromolecular studies had been started in 1976 as a joint project with Professor Richard R. Ernst (Nobel Prize in Chemistry, 1991). In 1977 the first 2D NMR spectrum of a protein was recorded, and by 1980 we had assembled four 2D NMR experiments that allowed for the initial protein structure determinations: COSY (2D correlated spectroscopy), SECSY (2D spin-echo correlated spectroscopy), FOCSY (2D foldover-corrected correlated spectroscopy), and NOESY (2D nuclear Overhauser enhancement spectroscopy). It was a lot of fun at the time to decide on these acronyms! Soon my group started to use 2D NMR experiments in daily practice, and the experience from more than a decade of one-dimensional NMR spectroscopy with proteins was happily and profitably married with the new potentialities of 2D NMR.

By 1982, complete sequence-specific assignments had been obtained for a small protein, BPTI, and for the polypeptide hormone glucagon bound to lipid micelles. This was published in a series of four papers in 1982. Although the first one of these papers already outlines the presently used protocol for protein structure determination by NMR, it took two more years of intense work on metric matrix distance geometry algorithms and their implementation in efficient software packages before the first NMR structure determination of a globular protein, bull seminal protease inhibitor (BUSI), could be completed. A large number of brilliant junior scientists working in my group from 1976 to 1985 contributed directly or indirectly to this result: Gerhard Wagner was involved in each step of the project; Kuniaki Nagayama and Peter Bachmann devised the first generation of 2D NMR experiments for studies of biological macromolecules and wrote the software needed to handle such data with the then-available limited computational facilities; Anil Kumar recorded the first 2D NOESY experiment with a protein; Gerhard Wider made key contributions to 2D NMR spectroscopy and to the sequential assignment method; Werner Braun, Martin Billeter, and Timothy Havel started a tradition in my laboratory of theoretical work on the structural interpretation of NMR data; Peter Strop prepared BUSI and worked on its resonance assignments; finally, Michael Williamson and Timothy Havel actually solved the structure of BUSI. They all, and many additional students and



The first two-dimensional ^{1}H NMR spectrum of a protein, a "2D J-resolved spectrum", recorded by Kuniaki Nagayama, 1977.

postdoctoral associates from the "heroic period" 1976–1985 have in the meantime started highly successful independent academic careers.

The completion of the first NMR structure of a protein brought new, unexpected challenges. When I presented the structure of BUSI in some lectures in the spring of 1984, the reaction was one of disbelief and suggestions that our structure must have been modeled after the crystal structure of a homologous protein. Apparently the structural biology community had thoroughly adjusted to the role of NMR as a method that could provide some exotic supplementary data, but which would not be suitable for de novo structure determination at atomic resolution. The criticism raised had two major consequences. The first one resulted from a discussion with Robert Huber (Nobel Prize in Chemistry, 1988), after a seminar in Munich, on May 14, 1984. Robert proposed to settle the matter by independently solving a new protein structure in his laboratory by X-ray crystallography and in my laboratory by NMR. For this purpose, each one of us received an ample supply of the α-amylase inhibitor Tendamistat from Hoechst AG. Virtually identical three-dimensional structures of Tendamistat were obtained by NMR in solution and by X-ray diffraction in single crystals, which settled matters once and for all. This was particularly comforting in the context of the fact that the subsequently solved NMR structure of metallothionein was completely different from an independently solved metallothionein crystal structure. (It took six years before the crystal structure was redetermined and found to coincide with the NMR structure!).

The second consequence was that I asked for a sabbatical leave and ended up in Wengen, a beautiful mountain resort in the Berner Oberland. This was possible because I was also finishing my 6-year term as the Secretary General of IUPAB in the summer of 1984. Considering the critical reaction to the initial NMR structure determinations, I felt that it was important to document our work in a complete and detailed fashion. I thus had good reasons to honor my commitment of writing a monograph on the Baker Lectures, which I had delivered in 1983 at Cornell University. As I spent much of the time alone in Wengen, with my family joining me for weekends and vacation periods, work progressed well. The book *NMR of Proteins and Nucleic Acids* covers primarily work in my research group during the period 1977–1984. It also turned out that directing my research group at a distance was surprisingly successful, and since the manuscripts were typed in Zürich from my hand-written notes, it helped that even ordinary mail was still reliably delivered within one day within Switzerland. It was therefore an easy decision for me to extend the stay in Wengen from the originally planned 6 months to 18 months. Besides the deskwork, important occupations in Wengen were skiing in the winter, and jogging and mountain climbing in the summer. According to my diaries I did not miss a single day of skiing from December 1, 1984 to April 10, 1985. This made up for having stayed away from the ski slopes during the 14 years from 1971 to 1984 because of my other professional activities. I also returned to the skiing outstation of the Federal Sports School in Mürren for a much-needed overhaul of my skiing technique, and participated in the organization of the famous Lauberhorn ski race in Wengen.

In the spring of 1986, after a second winter of skiing in Wengen, I had thoroughly cleaned up the backlog of unpublished material, in addition to having finished work on the Baker Lectures monograph. Protein structure determination by NMR had by then found its believers, as documented by the fact that the first printing of my new book was sold out within a few weeks. For us a new chapter had to be opened, and we established contacts with biochemists and molecular biologists for the real test of the NMR technique in applications to biologically interesting systems. By 1990, a collaboration with Professor Walter Gehring of the Biocenter at the University of Basel yielded structure determinations of the *Antennapedia* homeodomain and its complex with the operator DNA. Using this structure as a platform, additional NMR experiments provided entirely novel insights into the role of hydration water for specific DNA recognition. A NMR structure determination of the cyclophilin A–cyclosporin A complex was pursued as a joint project with two former graduate students, Hans Senn and Hans Widmer, and their research team at Sandoz AG. It had immediate practical impact, since the structure of the bound

Tim Havel and Mike Williamson in front of a computer displaying the three-dimensional structure of the protein BUSI that we had just solved, 1984.

View from my office in our home in Wengen, Switzerland, during my first-ever sabbatical, 1984–1986.

immunosuppressant turned out to be very different from the only other structural information available at the time, that is, crystal and NMR structures of free cyclosporin A. It was, for all involved, a completely unexpected and for many reasons surprising result!

In yet another exciting collaboration, with Professor Rudi Glockshuber at the ETH Zürich, we completed a structure determination for the C-terminal half of the mouse prion protein in April 1996, barely 10 days after the BSE-crisis in Great Britain broke into the open. With this timing, the prion protein structure had high visibility also in the popular media. In 1997 we succeeded in characterizing the structure of the intact prion protein, and found that the N-terminal half of the molecule forms a highly flexible, extended tail. The prion protein thus presented a striking illustration of the unique power of NMR to characterize partially structured polypeptide chains. Others among the more than 70 protein structure determinations completed in my laboratory functionally relate to enzymology, toxicology, chaperone-mediated protein folding, and intercellular signaling.

The biological and biomedical projects pursued during the past 16 years with the use of the NMR technique have added and still add greatly to the quality of my professional life. In these endeavors, the quite extreme specialization needed to maintain a high standard of structure determination breaks open in that I learn about an ever-increasing range of biological systems and biomedical problems. I feel very fortunate that my field of specialization thus leads me to an education in biology from the real experts, who sometimes even tend to consider me as one of their own.

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy is unique among the methods available for three-dimensional structure determination of proteins and nucleic acids at atomic resolution, since the NMR data can be recorded in solution. Considering that body fluids such as blood, stomach liquid, and saliva are protein solutions where these molecules perform their physiological functions, knowledge of the molecular structures in solution is highly relevant. In the NMR experiments, solution conditions such as the temperature, pH, and salt concentration can be adjusted so as to closely mimic a physiological fluid. Conversely, the solutions may also be changed to quite extreme nonphysiological conditions, for example, for studies of protein denaturation. Furthermore, in addition to protein structure determination, NMR applications include investigations of dynamic features of the molecular structures, as well as studies of structural, thermodynamic, and kinetic aspects of interactions between proteins and other solution components, which may either be other macromolecules or low-molecular-weight ligands. Again, for these supplementary data it is of keen interest that they can be measured directly in solution.

The NMR structure of the *Antennapedia* homeodomain (Figure 1) illustrates one of the exciting features of being able to perform structural studies in solution. The polypeptide chain in this protein is only partially folded, with both chain ends showing pronounced disorder.[1] In the complex with its operator DNA (Figure 2) the *N*-terminal chain end is located in the minor groove of the DNA, where it adopts a well-defined structure.[2] This mode of intermolecular recognition illustrates the functional importance of partially structured polypeptide chains. Mammalian prion proteins are an even more striking example of partial polypeptide folding, since the three-dimensional structure of the benign cellular form (PrP^C) includes a flexibly disordered 100-residue tail linked to the N-terminal end of a globular domain (Figure 3).[3] Considering that the mechanism of transformation of PrP^C into the aggregated, disease-related form of mammalian prion

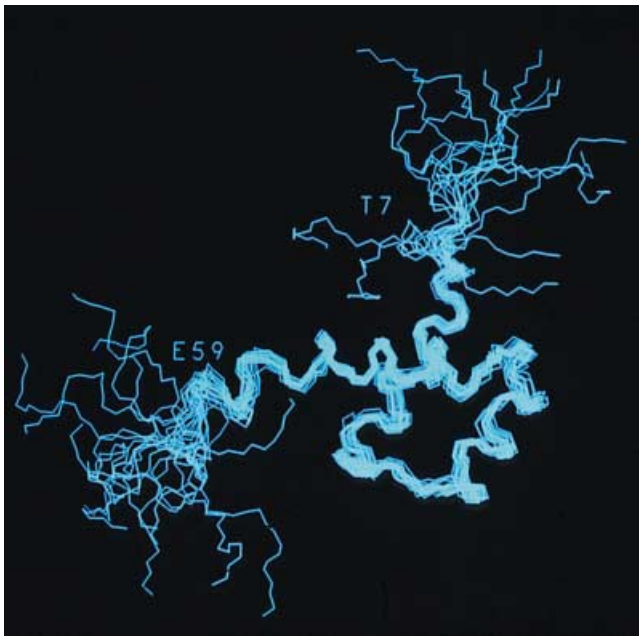


Figure 1. NMR structure of the *Antennapedia* homeodomain. A bundle of 20 superimposed conformers represents the polypeptide backbone. For the polypeptide segment 7–59, the tight fit of the bundle indicates that the structure is defined with high precision, whereas the two chain ends are disordered.

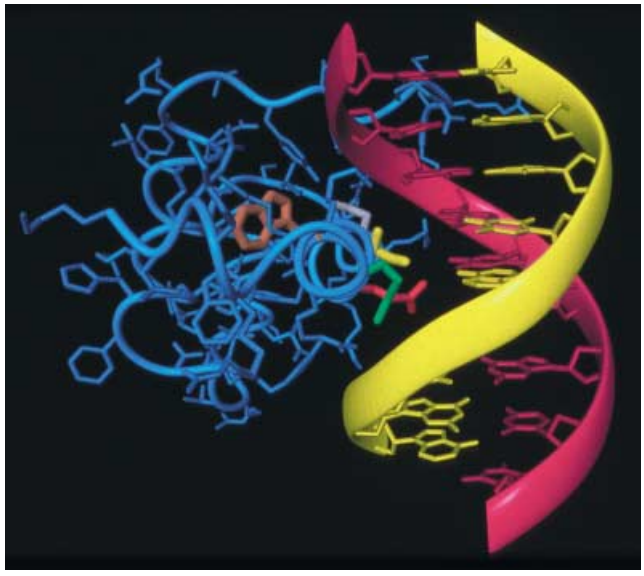


Figure 2. NMR structure of the complex formed between the *Antennapedia* homeodomain (blue, with functionally important residues in red, yellow, green, and brown and its operator DNA (yellow and red).[2]

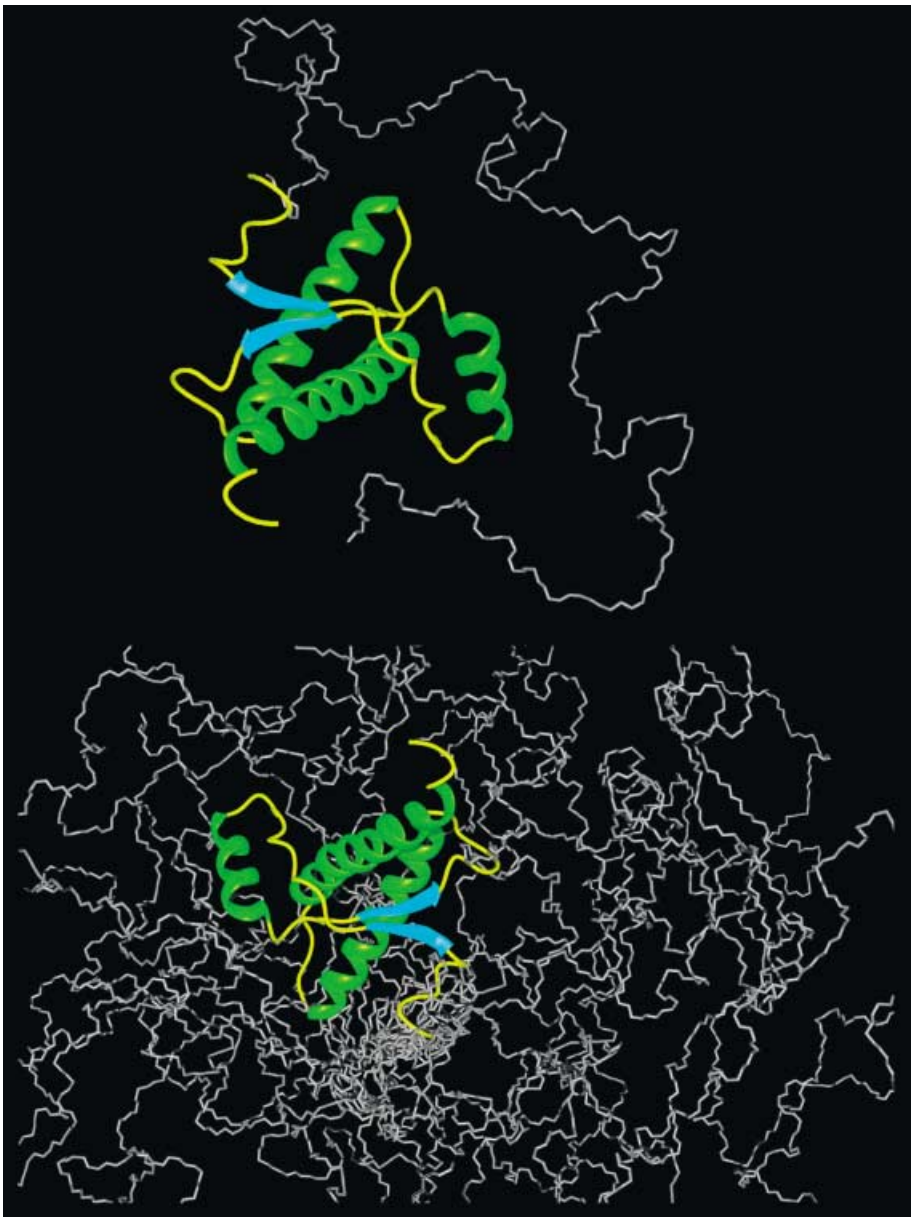


Figure 3. Top: NMR structure of the bovine prion protein. In the C-terminal globular domain of residues 126–230, α helices are green, an antiparallel β sheet is blue, and nonregular secondary structure is yellow; the "unstructured" N-terminal tail of residues 23–125 is white. Bottom: Visual impression of the variation of the bovine prion protein structure during a time period of about 1 ns. The superposition of 20 snapshots illustrates that the globular domain maintains its mean geometry, whereas the tail undergoes large-scale changes with time.

proteins is still subject to speculation, the observation of this flexible tail has been highly intriguing.

Partially folded polypeptide chains are usually difficult to crystallize. Furthermore, if crystals can be obtained, the chain segments that are disordered in solution will either be ordered by intermolecular contacts in the crystal lattice, or they will not be visible by diffraction methods. As a consequence, NMR has in many cases been the only method capable of providing structural information on partially folded polypeptides. Although a standard protocol for NMR structure determination provides a static picture of the unstructured chain segments (see Figures 1 and 3),[4] additional NMR experiments can provide information on the frequencies of the rate processes that mediate transitions between discrete states of the molecule within the conformation space spanned by the static bundle of NMR conformers.[5,6]

The ability of NMR techniques to characterize macromolecular structures and their intermolecular interactions with high spatial and temporal resolution has long attracted keen interest. This article reports on experience gained with NMR studies of proteins in my laboratory during the past 35 years.[7]

2. NMR Spectra of Proteins in Solution

When I joined the field of biomacromolecular NMR spectroscopy in 1967, important qualitative NMR features of amino acids and proteins had already been noted and tentatively rationalized.[8–12] Most important, it had been well documented that the spectrum of a globular protein is more complex than the sum of the NMR lines from the constituent amino acid residues in the polypetide chain (Figure 4a), and the differences between the ^{1}H NMR spectra of folded and unfolded (random coil (rc)) forms of a polypeptide chain had been tentatively explained by different interactions with the solvent (Figure 4b). The spectral analysis was primarily focused on the positions of the individual NMR lines in the ^{1}H NMR spectrum, as given by the chemical shift, δ, in parts per million (ppm) relative to a reference compound (Figure 4a). Although the chemical shift is primarily determined by the covalent structure of the amino acid residue, it can also be significantly affected by the interactions with the solvent. Therefore, the exclusion of the solvent water from the interior of a globular protein (Figure 4b) causes the chemical shifts of the core residues to be different from those of the water-exposed amino acid residues, so that even NMR lines originating from multiple residues of the same amino acid type can be distinguished. This conformation-dependent chemical shift dispersion was found to be sufficiently large to enable ^{1}H NMR studies of protein denaturation (Figure 5).[12] This then indicated the exciting prospect of using NMR for detailed studies of protein folding, and in particular for distinguishing between two-state and multiple-state folding and unfolding transitions. For me, the observations and ideas illustrated by Figures 4 and 5 also showed that one would need the ability of resolving and analyzing highly complex NMR spectra (upper trace in

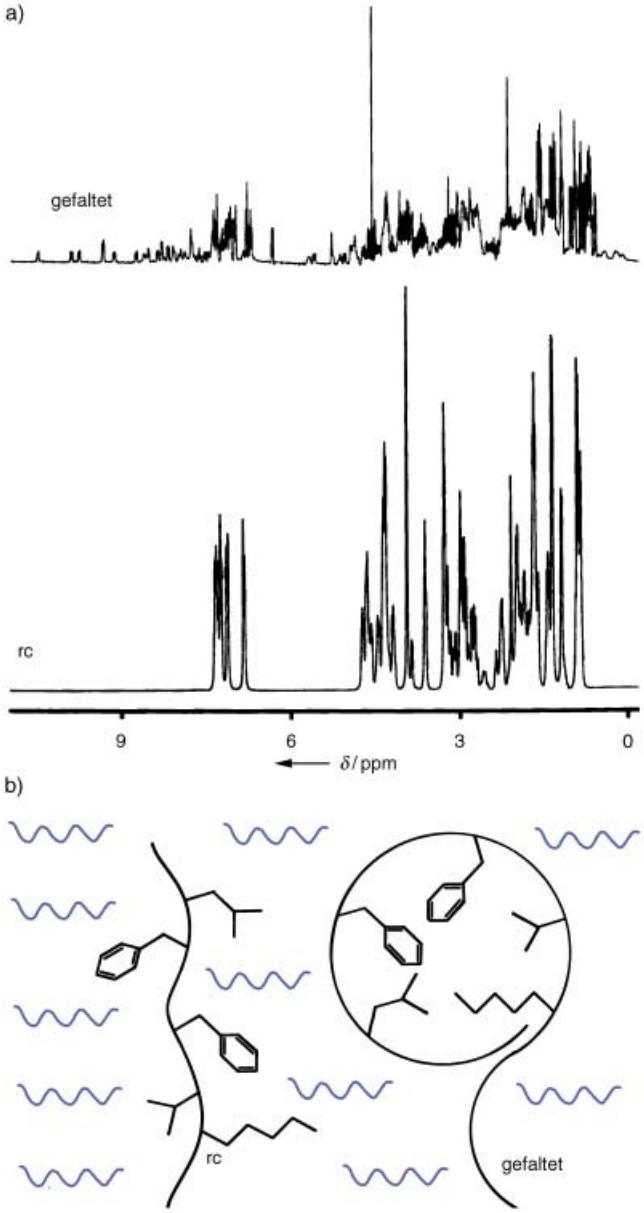


Figure 4. a) One-dimensional (1D) ^{1}H NMR spectra of the small protein bovine pancreatic trypsin inhibitor (BPTI, $M \approx 6000$). Top: Experimental spectrum of folded, active BPTI in a freshly prepared ^2H_2O solution. Bottom: Simulated spectrum for the unfolded, random coil form of the BPTI polypeptide chain. B) The solvent (blue) has free access to all parts of a random coil polypeptide chain (rc), whereas it is excluded from the core of a folded globular protein.

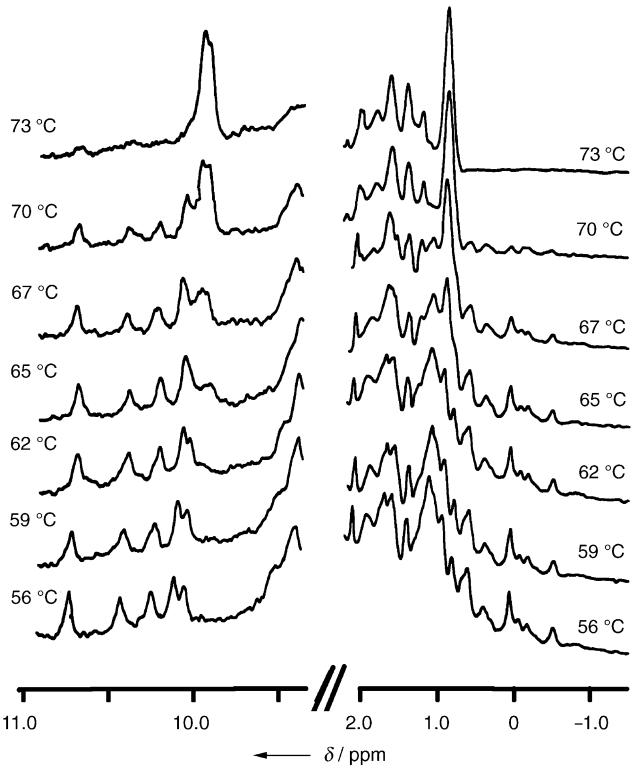


Figure 5. Downfield and upfield regions of 1D ^{1}H NMR spectra of the protein lysozyme at different temperatures, reflecting the transition from the folded form at 56 °C to the random coil form of the polypeptide chain at 73 °C (220 MHz, H_2O solution)[12].

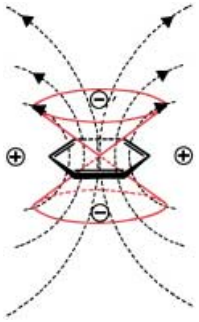


Figure 6. Local "ring-current field" around aromatic rings in solution induced by an external, static magnetic field. The shape of the ring current field is indicated by the red double-cone and by broken magnetic-field lines. The minus sign indicates that the NMR lines of hydrogen atoms located inside the cone in the three-dimensional protein structure are shifted upfield (to the right in the spectra of Figure 4a), whereas for atoms outside of the cone the shifts are downfield.

Figure 4a) in order to obtain information about physiologically active, folded forms of proteins. This was a clearly defined problem that appeared sufficiently challenging to be attractive for my own research.

In attempts at rationalizing in more detail the observations of Figure 4a and Figure 5, it had been suggested that local magnetic fields generated by ring currents in the aromatic amino acid side chains (Figure 6) should cause outstandingly large conformation-dependent chemical-shift changes of hydrogen atoms located near the rings in the three-dimensional protein structure. This was qualitatively confirmed by comparison of the largest observed deviations from random coil chemical shifts[4] in hen egg white lysozyme (Figure 5) with calculations of the ring-current shifts based on the then-available low-resolution crystal structure of this protein.[12] It had also been pointed out that hemoproteins could be expected to have particularly large ring-current shifts for hydrogen atoms located near the heme groups (Figure 7).[10,12]

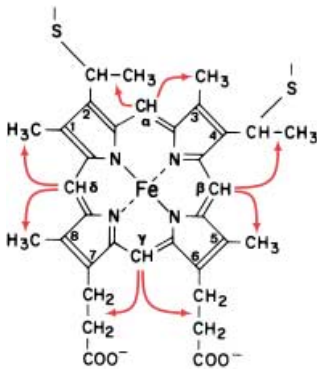


Figure 7. Chemical structure of heme *c*, which is the prosthetic group in cytochromes *c*. The red arrows connect groups of hydrogen atoms in the covalent structure of heme *c* that are separated by a sufficiently short distance to be connected by nuclear Overhauser effects (NOE).

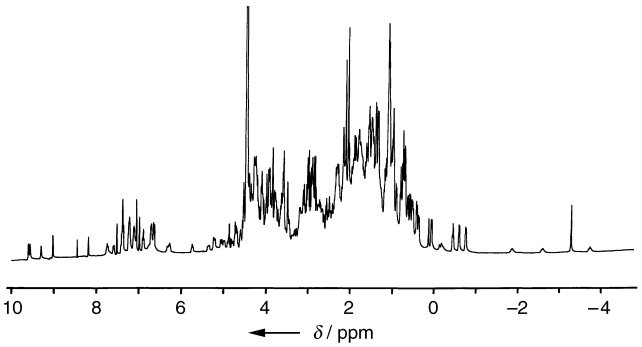


Figure 8. 1D ^{1}H NMR spectrum (360 MHz, 52 °C, solvent $^{2}H_2O$) of horse ferrocytochrome *c* ($M \approx 12000$).

For my start in the field of NMR with proteins, the prediction of unique, large heme ring-current shifts seemed to be an attractive feature of hemoproteins, and the presence of the heme iron (Figure 7) was appealing in view of my background in inorganic chemistry. As an additional advantage, low-resolution crystal structures for several hemoproteins were already available in 1967. It turned out that for several years nearly all my research projects were focused on the heme iron and its coordinatively linked ligands, and on the nonbonding interactions of the heme groups with their immediate environment in the hemoproteins. As anticipated, the large heme ring-current field generates a small number of well-separated resonance lines in the spectra of folded, globular hemoproteins (Figure 8). For the paramagnetic states of hemoproteins, additional well-resolved lines result from interactions with the unpaired electrons of the heme iron. Although they represented less than 3% of all the hydrogen atoms in any given hemoprotein, the well-resolved resonances were of crucial importance, because these lines could be selectively irradiated in 1D ^{1}H NMR experiments. For example, performing some simple ligand exchange and redox reactions with cytochrome *c* resulted in the identification of one of the axial ligands bound to the heme iron, which had not been seen in the low-resolution X-ray crystal structure available in 1969 (Figure 9).

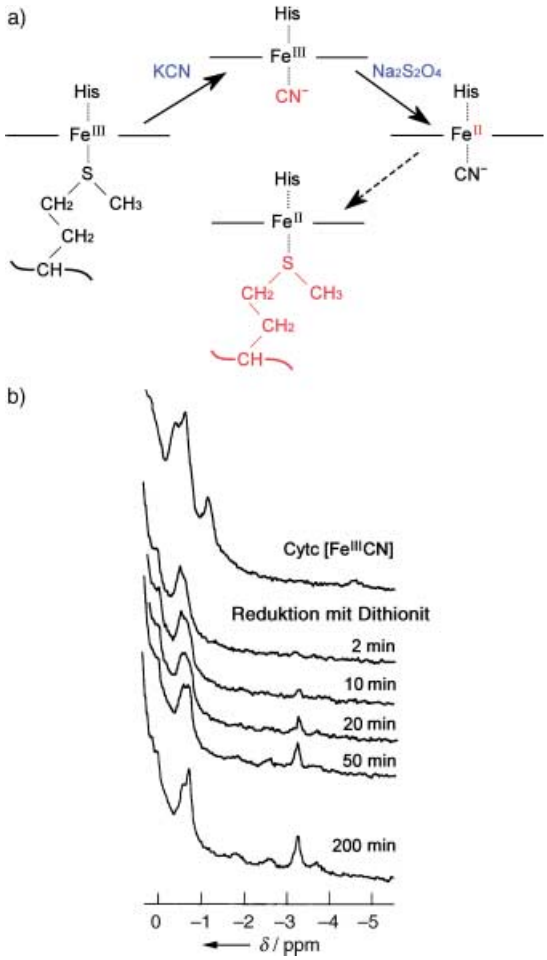


Figure 9. a) Three chemical reaction steps with cytochrome *c* that were used to identify methionine as one of the axial ligands of the heme iron.[13] The roman numerals indicate the oxidation state of the heme iron, and His stands for the amino acid histidine. The solid and broken arrows indicate rapid and slow reaction steps, respectively. Black: heme iron with ligands, where the horizontal line through Fe represents a side view of the heme *c* ring (see Figure 7); blue: chemicals added to the protein solution; red: new structural features resulting from the preceding reaction. b) Changes with time in the 1D ^{1}H NMR spectrum of cytochrome *c* after reducing the ferric cyanide complex with dithionite (220 MHz, 9 °C, solvent $^{2}H_2O$).

The experiments in Figure 9a[13] started with the oxidized form of cytochrome *c*. As was then learned through this study (see below), ferricytochrome *c* contains a methionine side chain in one of the axial heme iron coordination sites. Upon addition of KCN to the protein solution, this methionine is replaced in the binding site on the heme iron by a cyanide ion, and therefore moves out of the heme ring-current field. The completion of this reaction can readily be monitored, since different patterns of well-resolved resonance lines are present in the ^{1}H NMR spectra of ferricytochrome *c* and its cyanide complex.[13] After reduction of the heme iron to the ferrous state, the cyanide complex is thermodynamically less stable than the native form of cytochrome *c*, and in a slow reaction the cyanide in the axial coordination site of the heme iron is again replaced with the natural methionine ligand. The return of the axial methionine into the heme ring-current field was

monitored in real time (Figure 9b) by the appearance of its typical high-field lines in the ^{1}H NMR spectrum (see Figure 8). This experiment resulted in the identification of the nature of this axial ligand in the native, active form of cytochrome *c*, and further yielded information on the relative thermodynamic stabilities of the four different structures in Figure 9a.

As a sideline, comparison of the Figures 8 and 9b also illustrates the influence of various experimental factors on the appearance of the NMR spectra. The higher resonance frequency and the high temperature used in the experiment of Figure 8, which was recorded around 1980 with Fourier transform spectroscopy, resulted in narrower and more clearly separated peaks in the region 0 to −5 ppm than the experiment in Figure 9b, which was recorded in 1968 with continuous-wave NMR spectroscopy at low temperature.

Other early experiments based on the observation of well-resolved resonances in the ^{1}H NMR spectra of hemoproteins resulted in the characterization of conformation changes during the oxygenation of myoglobin and hemoglobin,[14,15] and in detailed characterization of the electronic structure of the heme groups in different classes of hemoproteins.[16–18]

3. The Way to NMR Structures of Proteins

Around 1970 there was a lot of enthusiasm in my laboratory and elsewhere about the success of experiments of the types illustrated by Figures 4–9. During the following years, however, there was little further progress toward an NMR method for de novo structure determination of proteins. In hindsight this can readily be rationalized: Early successful structural interpretations of NMR data invariably supplemented a previously known low-resolution X-ray crystal structure of the same protein. In spite of the high symmetry of the ring-current fields (Figure 6) and other sources of local conformation-dependent chemical shifts, including natural paramagnetic centers and extrinsic paramagnetic shift reagents,[17,19] the observed chemical shifts could thus in some instances yield exciting new information. In a de novo protein structure determination, however, the high symmetry of the local magnetic fields would lead to ambiguities in the structural interpretation of the resulting chemical shifts. Different approaches were therefore called for, and eventually a NMR method for protein structure determination could be based on the following four principal elements:

3.1 Measurement of NOE Upper Distance Limits as Conformational Constraints

Nuclear Overhauser effects (NOEs) are due to dipolar interactions between different nuclei. The intensity of the NOE is related to the product of the inverse sixth power of the internuclear distance and a correlation function, $f(\tau_c)$, which describes the modulation of the dipole–dipole coupling by stochastic rate processes, with an effective correlation time τ_c [Eq. (1)].

$$\mathrm{NOE} \propto \frac{1}{\langle r \rangle^6} f(\tau_c) \qquad (1)$$

Although the NOE is a common phenomenon for all combinations of closely spaced nuclear spins, NOEs between pairs of hydrogen atoms are of prime interest for structural studies. A ^{1}H–^{1}H NOE is related to the through-space distance between a pair of atoms that are either not at all linked by covalent bonds (intermolecular NOE), or that may be far apart in the amino acid sequence of a polypeptide chain. ^{1}H–^{1}H NOEs can be observed in double-irradiation 1D NMR experiments as the fractional change in intensity of an NMR line that results from preirradiation of another resonance (Figure 10). Although NOE distance measurements had successfully been used for studies of small organic molecules,[20] including oligopeptides,[21] and observation of NOEs in proteins had also been reported,[22,23] it was not clear whether the desired distance information could actually be obtained for proteins.[20,24,25] This uncertainty arose because the Brownian motions of large structures in solution are slow, with long effective correlation times, τ_c, for this stochastic process, and proteins contain a dense network of hydrogen atoms. With the combination of these two features, spin diffusion could partially or fully deteriorate distance measurements based on ^{1}H–^{1}H NOE experiments.[24–28]

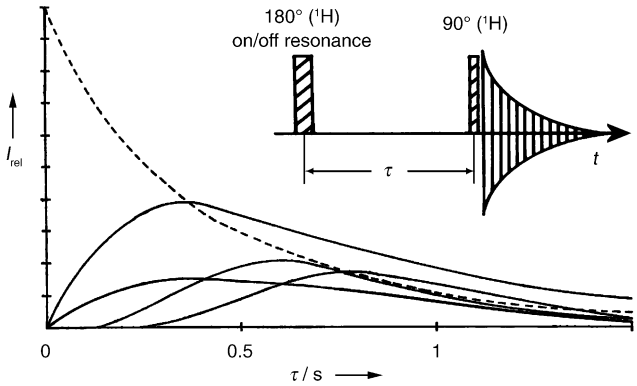


Figure 10. NOE buildup curves observed with 1D transient [^{1}H,^{1}H]-NOE measurements.[26] The relative intensities, I_{rel}, of the preirradiated resonance line (broken curve) and of the lines experiencing NOEs (solid curves) are plotted versus the duration of the mixing period, τ. The experimental scheme used to record transient NOE spectra (inset in the upper right) includes a selective 180° radio-frequency pulse applied to the "pre-irradiated" ^{1}H NMR line and a nonselective 90° pulse applied to the entire ^{1}H NMR spectrum. The two pulses are separated by the mixing period, τ, and followed by signal acquisition. When working with the crowded spectra of proteins, one records the difference between two spectra obtained with the preirradiation 180° (^{1}H)-pulse applied on- and off-resonance.

In the solution NMR experiments discussed here, spin diffusion arises as a consequence of the dependence of the NOE on the inverse sixth power of the internuclear distance, since magnetization transfer between two spins through multiple short steps may be more efficient than a one-step transfer over the longer, direct distance (Figure 11). We used 1D transient NOE-difference experiments (inset in

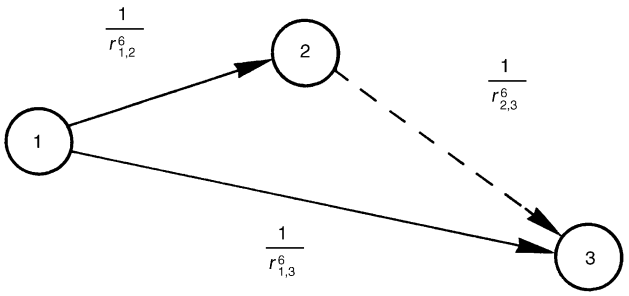


Figure 11. Spin diffusion: Transfer of magnetization between two hydrogen atoms 1 and 3 in the presence of additional hydrogen atoms goes through two competing pathways: the direct NOE across the distance $r_{1,3}$ and two- or multiple-step spin diffusion via intervening hydrogen atoms.

Figure 10)[26] and 1D truncated-driven NOE-difference experiments[27] to record NOE buildup curves (Figure 10), which then provided a basis for ^{1}H–^{1}H distance measurements in macromolecules. These studies showed that even more favorable conditions for NOE distance measurements can be found in macromolecules, which have long effective correlation times for the modulation of dipole–dipole couplings, than in low-molecular-weight compounds, for which the condition of extreme motional narrowing applies.[24,28]

Analysis of ^{1}H–^{1}H NOE buildup curves recorded with 1D transient NOE experiments (Figure 10) is based on the consideration that during the early phase of the mixing period, direct magnetization transfer from spin 1 to spin 3 (Figure 11) will result in a linear increase of the magnetization on spin 3 with time. In contrast, transfer to atom 3 via an intermediate step through atom 2 will have a lag period, even though for long mixing periods, τ, the extent of the magnetization transfer by such spin diffusion may for individual pairs of hydrogen atoms exceed that of the direct transfer (Figure 10). Corresponding considerations apply for the analysis of NOE buildup curves recorded with truncated-driven NOE experiments.[27] With proper selection of the duration of the mixing period, one can thus measure highly selective ^{1}H–^{1}H NOEs between distinct pairs of hydrogen atoms, or groups of chemical-shift-equivalent hydrogen atoms in proteins in solution.

There is a second criterion that needs to be satisfied for obtaining selective NOEs with 1D NMR experiments, that is, at least one of the two lines that are connected by the NOE must be sufficiently well resolved (i.e., separated from all other lines in the spectrum) to enable selective radio-frequency preirradiation (inset in Figure 10). The previously discussed well-resolved lines in the ^{1}H NMR spectrum of ferrocytochrome *c* (Figure 8) thus had again an important role in enabling us to study the spin physics in the interior of this globular protein with 1D ^{1}H NMR experiments, as well as to obtain novel structural information. Figure 12 shows a series of highly selective 1D truncated-driven NOE measurements, which were used to determine ^{1}H NMR assignments for the heme group in a *c*-type cytochrome (see Figure 7).[29]

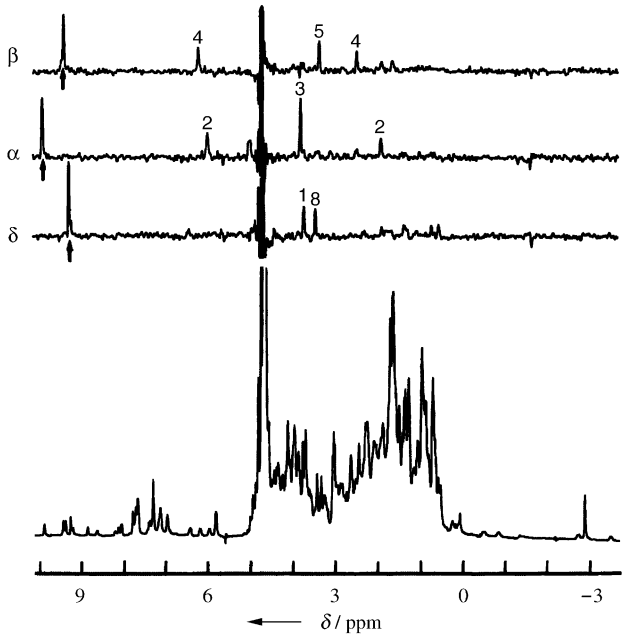


Figure 12. ^{1}H NMR assignments for the heme group of a *c*-type cytochrome using ^{1}H–^{1}H NOEs (see Figure 7). Bottom trace: 1D ^{1}H NMR spectrum of ferrocytochrome *c*-551 from *Pseudomonas aeruginosa* ($M \approx 12000$). Upper traces: three 1D truncated-driven NOE-difference spectra obtained with selective preirradiation (indicated by arrows) on the hydrogen atoms β, α, and δ (360 MHz, 35 °C, ^2H_2O solution). The NOE peaks are identified with numbers indicating the substituents attached to the corresponding porphyrin ring carbons (see Figure 7).

3.2 Sequence-Specific Resonance Assignments

Similar to the situation in heme groups (Figure 7), there are closely spaced pairs of hydrogen atoms in neighboring residues of a polypeptide chain (Figure 13). These can be connected by the observation of sequential NOEs. Figure 13 illustrates that NOE-based ^{1}H NMR assignments for a polypeptide chain can conceptually be considered as a two-step process. Each amino acid residue represents a spin system,[4] that is, it consists of an array of hydrogen atoms, including an amide proton (H^N), an α-proton (H^α), and the side-chain protons, which can be connected by steps over three or less

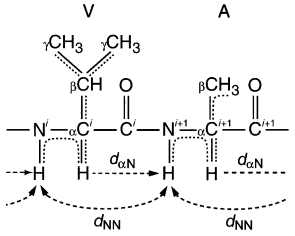


Figure 13. Sequential ^{1}H NMR assignment of proteins. The drawing shows the chemical structure of a valine–alanine (V–A) dipeptide segment in a polypeptide chain. The dotted lines connect groups of hydrogen atoms that are separated by at most three chemical bonds and can therefore be connected using scalar spin–spin couplings. The broken arrows link pairs of hydrogen atoms in neighboring amino acid residues that are separated by short through-space distances, $d_{\alpha N}$ and d_{NN}, and can therefore be connected by "sequential NOEs".

covalent bonds through the observation of scalar spin–spin (through-bond) couplings (see reference [4] for the exceptions represented by proline and the aromatic side chains). In contrast, hydrogen atoms located in sequentially neighboring amino acid residues are separated by at least four covalent bonds. Pairs of neighboring residues in the sequence can therefore only be connected via NOEs manifesting short through-space distances, such as $d_{\alpha N}$ and d_{NN}. Suitable combinations of intraresidual ^1H–^1H connectivities established by scalar spin–spin couplings, and interresidue connectivities established by sequential NOEs enable progressive resonance assignments while "walking along the polypeptide backbone".

In other words, the spin systems of two neighboring residues can be connected by the intervening H^α–H^N or H^N–H^N sequential NOE connectivities (Figure 13). 1D double-resonance experiments with selective irradiation of the well-resolved amide proton resonances between 8 and 10 ppm in folded BPTI (Figure 4a) thus yielded assignments for most of the residues in the regular secondary structure elements (Figure 14).[30] Further assignments were not possible, because the other regions of the polypeptide chain were not represented by well-separated ^1H NMR lines that could have been selectively irradiated in 1D NMR experiments.

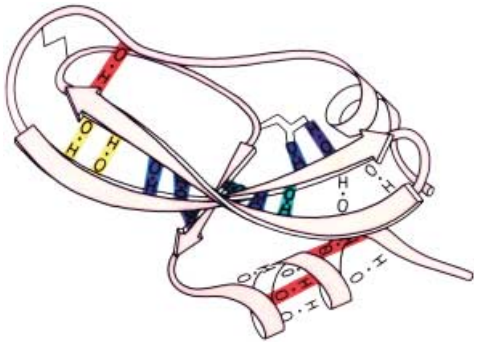


Figure 14. Sequence-specific resonance assignments for BPTI that were obtained using 1D ^1H NMR experiments.[30] The polypeptide backbone is shown, and the assigned residues are identified by indication of hydrogen bonds with their amide protons (the color code indicates variable exchange rates of the amide protons with the solvent; drawing by Jane Richardson, 1980).

3.3 Two-Dimensional (2D) NMR

With the introduction of 2D NMR experiments, and subsequently 3D and 4D NMR experiments, NMR studies of biological macromolecules evolved from intellectually stimulating science to a practical approach for protein structure determination. The foundations of multidimensional NMR have been presented in the Nobel Lecture by Richard R. Ernst.[31] Here, I only want to comment on two crucial consequences of multidimensional NMR for studies of proteins. First, 2D NMR enables the recording of selective interactions between pairs of hydrogen atoms, or groups of chemical-shift-equivalent hydrogen atoms, without selective irradiation of individual resonance lines. It thus enables a detailed analysis of the entire ^1H NMR spectrum of a protein, which contrasts with the 1D NMR situation of being limited to using only a small number of resolved lines at the periphery of the spectrum. Second, the dispersion of the resonances in a two-dimensional frequency plane affords greatly improved separation of the individual peaks. For example, in Figure 15

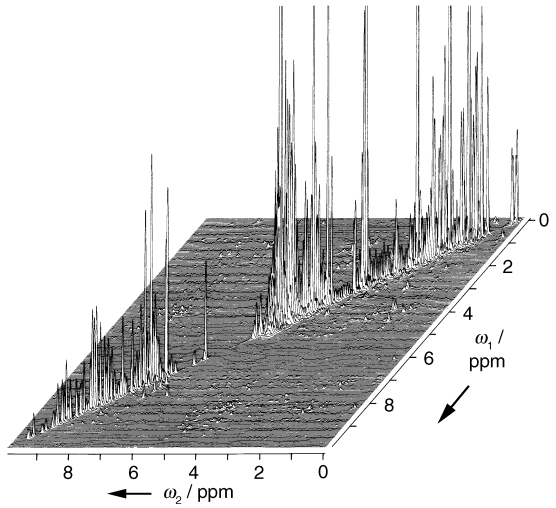


Figure 15. Two-dimensional (2D) [^1H,^1H]-NOE spectroscopy ([^1H,^1H]-NOESY). A stacked plot representation of a spectrum of the small protein bull seminal proteinase inhibitor IIA (BUSI IIA, $M \approx 6000$) is shown (500 MHz, 45 °C, H_2O solution).

the intense lines on the diagonal from the lower left to the upper right correspond to the 1D NMR spectrum, and the weak cross-peaks in the plane outside of the diagonal manifest selective NOEs between pairs of hydrogen atoms, which are represented in the NMR spectrum by two distinct (but not necessarily resolved) chemical shift positions along the diagonal.

The greatly improved separation of the individual cross-peaks is best seen in contour plots of 2D NMR spectra (Figure 16), which is the presentation used for detailed analysis. The 1D decoupling experiments for the identification of scalar spin–spin couplings were thus replaced by 2D correlation experiments, such as COSY, SECSY, and FOCSY, and the 1D NOE-difference experiments were replaced by 2D [^1H,^1H]-NOESY, which yielded similar NOE buildup curves for each NOESY cross-peak (Figure 17) as the 1D transient NOE technique (Figure 10).[4,32,33] Using these 2D ^1H NMR experiments, complete sequence-specific resonance assignments could be obtained for the protein BPTI (Figure 18).[34]

The NOESY–COSY connectivity diagram in Figure 19[4,35] makes use of the fact that standard 2D [^1H,^1H]-COSY and 2D [^1H,^1H]-NOESY spectra are symmetrical with respect to the diagonal peaks. Combining the upper left half of a NOESY spectrum with the lower right half of a COSY spectrum therefore enables a straightforward visualization of assignments by a succession of $d_{\alpha N}$ sequential NOEs and intraresidual H^N–H^α scalar coupling connectivities. The data were recorded in a freshly prepared solution of BPTI in ^2H_2O, where only the resonances of the slowly

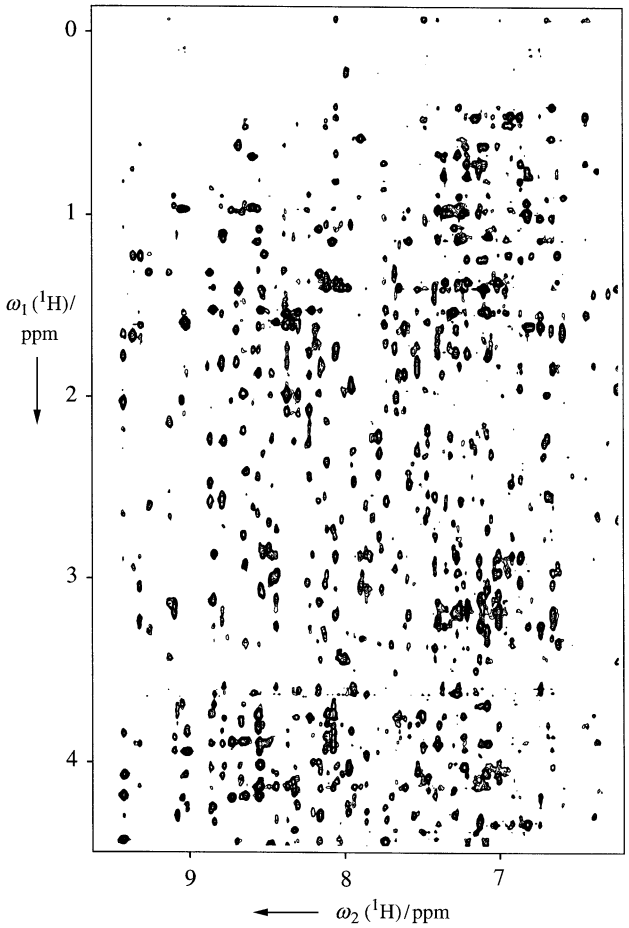


Figure 16. 2D [^{1}H,^{1}H]-NOESY spectrum of the plant pathogenesis-related protein P14A ($M \approx 15000$). A contour plot of the spectral region $\omega_1(^1H) = 0$–4.3 ppm, $\omega_2(^1H) = 6.3$–9.5 ppm (750 MHz, 30 °C, H_2O solution) is shown.

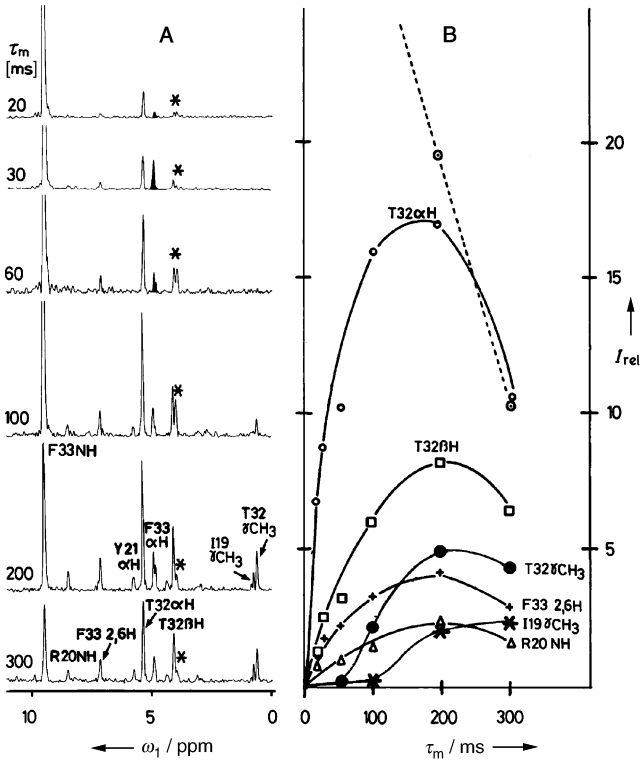


Figure 17. Measurement of NOE buildup curves using 2D [^{1}H,^{1}H]-NOESY experiments. A) Six NOESY spectra of BPTI recorded with different mixing times, τ_m. The same 1D cross-section along the ω_1 frequency axis through the diagonal peak of the Phe 33 amide proton is plotted for each spectrum. B) NOE buildup curves obtained from the analysis of the data in (A); same presentation as in Figure 10, with the relative intensities of the NOESY cross-peaks plotted versus the mixing time, τ_m, and the broken line representing the decay of the magnetization on the diagonal peak of the Phe 33 amide proton.

exchanging amide protons are seen.[35] The assignments start with the COSY cross-peak identified by a black square and, as indicated by the arrows, go clockwise to the sequentially preceding isoleucine residue, and counterclockwise to a sequence of four residues. It is customary that the data leading to the ^{1}H NMR assignments of a protein are collected in a plot versus the amino acid sequence (Figure 20). The figure shows that most of the sequential connectivities are independently documented by two or three different sequential NOEs,[4,36] and that possible remaining gaps in the assignment pathway are readily recognized in this presentation.

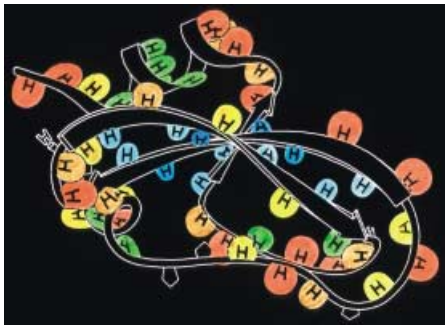


Figure 18. Complete sequence-specific resonance assignments for BPTI obtained using 2D NMR experiments.[34] Each assigned residue is identified by a colored patch around the amide proton (see also the legend to Figure 14; drawing by Jane Richardson, 1982).

3.4. Structural Interpretation of NOE Distance Constraints.

A polypeptide chain with 100 amino acid residues has a length of about 400 Å, whereas NOE-observable distances are shorter than about 5 Å. Observation of a NOE between a pair of hydrogen atoms with assigned chemical shift positions therefore enforces the formation of a ringlike structure (Figure 21). A successful structure determination generates three-dimensional arrangements of the polypeptide chain that simultaneously contain all the small and large circular structures imposed by the ensemble of all NOESY cross-peaks.

Partial structure determination has been obtained by an empirical approach for the identification of regular secondary structures in polypeptide chains, which relies on recognizing distinct patterns of NOEs.[4,37] For example, in Figure 20 a

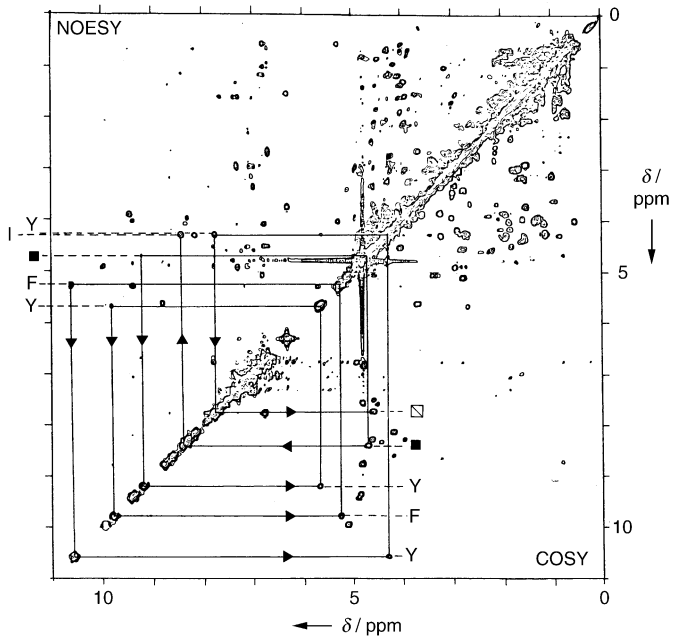


Figure 19. NOESY–COSY connectivity diagram for sequential ^{1}H NMR assignments, using the $d_{\alpha N}$ sequential NOEs. The data were recorded with BPTI in ^2H_2O solution (see text).

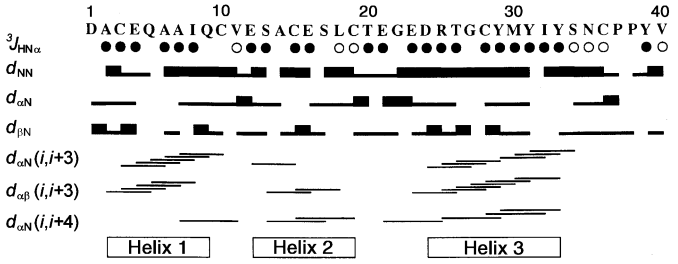


Figure 20. Standard presentation of ^{1}H NMR data leading to sequence-specific resonance assignments and the identification of regular secondary structures in proteins. Experimental data plotted versus the amino acid sequence are shown for the small pheromone protein Er-1 from *Euplotes raikovii* ($M \approx 4500$). $^3J_{HN\alpha}$ are scalar spin–spin coupling constants, with small and big values indicated by filled and empty circles, respectively. d_{NN}, $d_{\alpha N}$ and $d_{\beta N}$ are distances manifested in sequential NOEs, and strong and weak sequential NOEs are indicated by a thick or a thin line, respectively. Small values of the distances $d_{\alpha N}(i,i+3)$, $d_{\alpha\beta}(i,i+3)$, and $d_{\alpha N}(i,i+4)$ are observed by medium-range NOEs linking the given atom types between residues spaced as indicated in the parentheses and by the short horizontal lines. The locations of three α helices are indicated at the bottom.

succession of strong sequential d_{NN} NOEs in combination with the observation of medium-range NOEs in the same polypeptide segment identifies three α-helical structures, which are independently also indicated by successions of small values for the scalar spin–spin couplings $^3J_{HN\alpha}$.[4,38]

For the calculation of complete three-dimensional protein structures from NMR data,[39] it was quite clear from the outset that an input of quantitative NOE distance measurements would be difficult to obtain. The observed NOEs depend on the proton–proton distance, *r*, as well as on the effective rotational correlation times, τ_c [Eq. (1)]. Since for

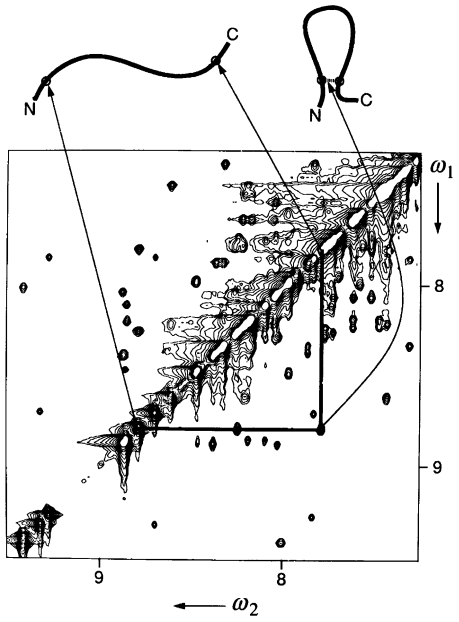


Figure 21. Scheme indicating the relations between an experimental 2D [^{1}H,^{1}H]-NOESY spectrum, a polypeptide with the chain ends indicated by N and C, sequence-specific assignments for two hydrogen atoms in the polypeptide chain indicated by circles, and the NOE upper distance constraint derived from the NOESY cross-peak connecting the chemical shift positions of two assigned hydrogen atoms (see text).

each pair of hydrogen atoms the effective τ_c-value is governed not only by the overall rotational molecular tumbling (Brownian motions), which depends on the size and shape of the protein as well as on the viscosity of the solvent, but can also be affected by intramolecular motions, $f(\tau_c)$ may vary for different pairs of hydrogen atoms in a protein molecule. Additional ambiguities could arise from partial quenching of individual ^{1}H–^{1}H NOEs by competitive spin-relaxation processes, for example, spin diffusion (Figure 11), chemical or conformational exchange, and interactions with other nuclear or electronic spins. Furthermore, as a result of rapid intramolecular mobility, a given NOE may be the result of sampling over a range of distances between the two hydrogen atoms of interest.[40]

In view of these intrinsic limitations for efficient quantitative NOE distance measurements, we decided to use a constant value of the correlation function, $f(\tau_c)$ [Eq. (1)], for all ^{1}H–^{1}H combinations in a protein, and to derive only upper limits on the ^{1}H–^{1}H distances from the NOE measurements. In practice, the input for a structure calculation then consists of allowed distance ranges, which are bounded by a NOE upper limit of 3.0–5.0 Å, depending on the intensity of the NOE, and a lower limit of 2.0 Å, which represents the sum of the van der Waals radii of the two NOE-connected hydrogen atoms. Although each individual entry in the input data thus has only limited precision, this procedure is robust and can conceptually account for the influence of intramolecular mobility in most of the situations that are commonly expected for the structured parts of globular proteins.

For the initial globular protein structure calculations from NMR data (Figure 22), we used a metric matrix distance

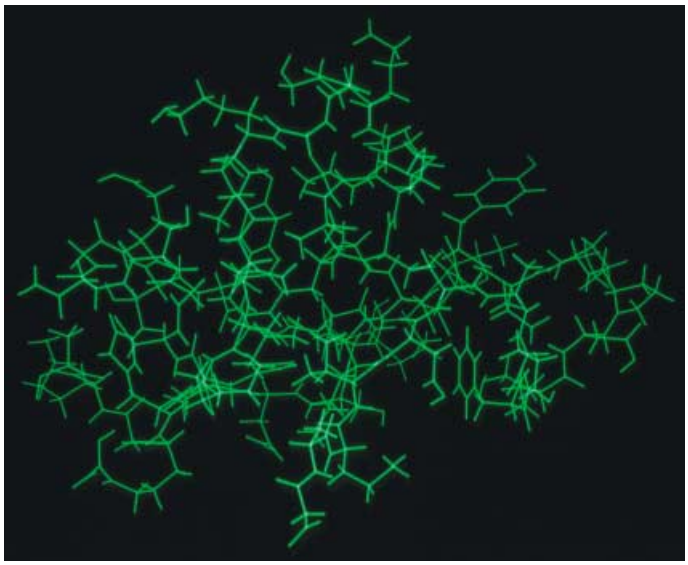


Figure 22. NMR structure of BUSI IIA.[43]

geometry algorithm to search for molecular geometries that are consistent with the ensemble of all experimentally determined NOE distance constraints.[41–43] Each such calculation ends with the minimization of an error function, and the residual error function value represents a straightforward measure for the success of having found a molecular geometry that satisfies the experimental input data. In view of the aforementioned distance-range format of the input, it is further of keen interest to evaluate the uniqueness of the calculated structure. To this end, the structure calculation is repeated with identical input data but different boundary conditions, and the uniqueness of the resulting NMR structure is judged from the tightness of the fit among the resulting ensemble of conformers. Typically, about 100 conformers are generated, and a subgroup of the 20 conformers with the smallest residual error function values is selected to represent the NMR structure of the protein. The average of the pairwise root-mean-square distances (RMSD) calculated for this bundle of conformers (Figure 1) is then taken as a measure for the precision of the structure determination. Visually, a tight fit of the bundle of conformers indicates regions where the structure is defined with high precision by the NMR data, whereas structurally disordered polypeptide segments show a large dispersion among the members of the bundle, as exemplified by the two chain ends of the *Antennapedia* homeodomain in Figure 1. In the absence of long-range NOE distance constraints, a properly functioning algorithm for the structure calculation will sample essentially all of the conformation space that is accessible with the given length of the polypeptide chain, as exemplified by the unstructured tail of the bovine prion protein in Figure 3 (bottom).

4. NMR in Structural Biology

4.1 Standard Protocol for NMR Structure Determination of Biological Macromolecules

The protocol for NMR structure determination includes the preparation of a homogeneous protein solution, the recording and handling of the NMR data sets, and the structural interpretation of the NMR data (Table 1). The techniques used in 1984 for the structure determination of bull seminal proteinase inhibitor IIA are listed in Table 1; the four steps of the structural interpretation (III in Table 1) were performed separately, although the result of the first round of constraint collection and structure calculation was subsequently used for additional checks on the sequence-specific resonance assignments as well as on the collection of conformational constraints. Since 1984, the protocol outlined in Table 1 has been used in over 3000 NMR structure determinations of proteins and nucleic acids, and greatly improved experimental techniques have been incorporated into this general scheme.

Table 1: Standard protocol for NMR structure determination of proteins.

Step[a]		BUSI IIA[b]
I	Sample preparation	Protein isolated from natural source; natural isotope distribution; 16 mM solutions in H_2O and in ^2H_2O
II	NMR spectroscopy	2D ^{1}H NMR
IIIa	Resonance assignments	Sequential NOEs
IIIb	Conformational constraints	[^{1}H,^{1}H]-NOEs, $^3J_{HN\alpha}$, $^3J_{\alpha\beta}$
IIIc	Structure calculation	Metric matrix distance geometry
IIId	Structure refinement	Restrained energy minimization

[a] The structural interpretation of the NMR data, step III, is somewhat arbitrarily divided up into four steps; in practice, one goes through multiple cycles of collection of conformational constraints (IIIb) and structure calculation (IIIc), and the completion of the sequence-specific assignments (IIIa) as well as the structure refinement (IIId) may also be part of this iterative approach. [b] This column lists the techniques used in the first structure determination of a globular protein in 1984.[43]

Major advances in the experimental techniques for NMR structure determination were spurred on by the introduction of methods for the production of recombinant proteins labeled with stable isotopes, in particular ^{13}C, ^{15}N, and ^{2}H.[44,45] For example, this opened the way for efficient use of heteronuclear NMR techniques with proteins, such as 3D [^{1}H,^{13}C,^{15}N]-triple resonance experiments, 3D ^{13}C- or ^{15}N-resolved [^{1}H,^{1}H]-NOESY (Figure 23),[46,47] and the use of heteronuclear filters.[48] Important advances have also been made with the methods of structure calculation, where the cpu time needed for the calculation of a small protein structure has been reduced from about one day in 1984[41,42] to a few seconds.[49] Currently, intense work is focused on the automation and combined execution of the individual steps in

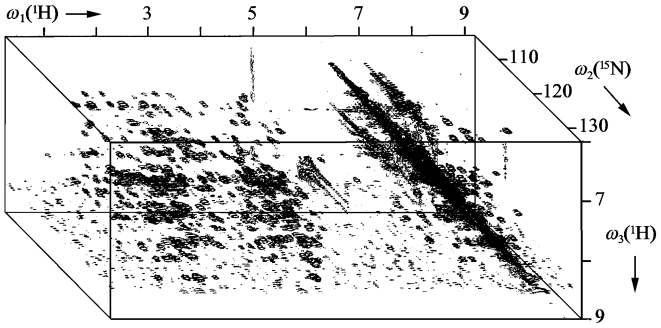


Figure 23. Three-dimensional (3D) ^{15}N-resolved [^{1}H,^{1}H]-NOESY spectrum (600 MHz, 28 °C, H_2O solution) of the DNA-binding domain of the P22 c2 repressor ($M \approx 10000$, uniformly ^{15}N-labeled).

the structural interpretation of the NMR data.[50,51] It is beyond the scope of this article to present in detail the wide range of beautiful novel experimental approaches developed by the community of macromolecular NMR spectroscopists during the past 15 years, which now enable studies with ever more complex systems (Figures 1–3).

4.2 Globular Protein Structures in Solution

The static picture of a protein molecule obtained from the standard protocol for structure determination (Table 1) typically shows variable precision of the structure determination along the polypeptide chain, as manifested by the variations in the closeness of the fit among the bundle of conformers used to represent the NMR structure (Figure 1). Even in proteins where the entire polypeptide chain is part of the global fold, increased disorder is observed toward the periphery of the surface side chains (Figure 24). This pronounced surface disorder, which typically also involves the ends of the polypeptide chain, is in most instances the only significant difference between corresponding globular protein structures in single crystals and in solution.[5] In part the increased disorder in solution arises because of a scarcity of packing constraints, when compared with the protein core, and a concomitant scarcity of NOE distance constraints. With the additional use of NMR spin-relaxation measurements,[24,28,33,52] one can distinguish between static disorder, which would then presumably arise from the scarcity of constraints, and dynamic disorder, with intramolecular motions on the nanosecond and sub-nanosecond time scale. Overall, quite independent of the dynamics issue, the observation of partially folded polypeptide chains in solution (Figures 1, 3 (bottom), and 24) is important complementary information to the data that can be obtained by studies in crystals. It is also the main reason why the quality of a NMR structure determination is not usually characterized by a single, global parameter.[4–6]

An important extension of the characterization of proteins in solution resulted from high-resolution NMR studies of protein hydration. Thereby the location of hydration waters is determined by the observation of NOEs between water protons and hydrogen atoms of the polypeptide chain.[53] Because of the dependence of the NOE on the inverse sixth power of the ^{1}H–^{1}H distance, only one layer of hydration water molecules is observed (Figure 25). For the hydration studies, the dependence of the NOE intensity on the correlation function describing the stochastic modulation of the dipole–dipole coupling between the interacting protons [Eq. (1)] has a key role. The value of $f(\tau_c)$ may be governed either by the Brownian rotational tumbling of the hydrated protein molecule, or by interruption of the dipolar interaction through translational diffusion of the water molecules relative to the protein surface, whichever is faster. On this basis it could be established that surface hydration of peptides and proteins is characterized by very short residence times of the water molecules in the hydration sites, in the range from about 20 to 300 picoseconds at 10 °C. This result presents an intuitive rationale for the generally observed dynamic disorder of the protein surface structure (Figure 24), and

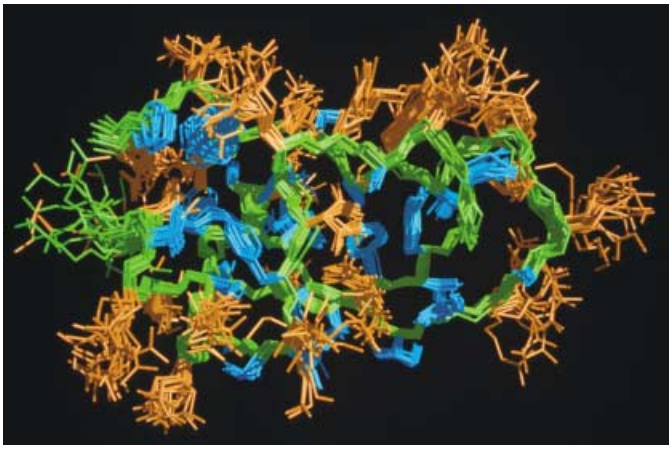


Figure 24. NMR structure of BPTI represented by a bundle of 20 conformers superimposed for best fit of the polypeptide backbone. The polypeptide backbone is green, core side-chains are blue, and solvent-accessible surface side chains are red.

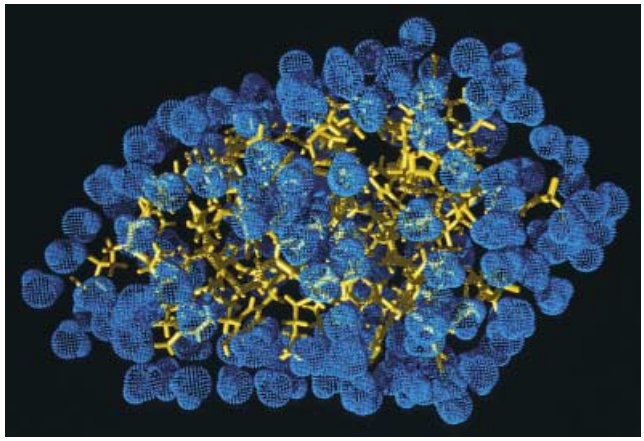


Figure 25. Molecular model of hydrated BPTI in H_2O solution. The drawing shows an all-heavy-atom-presentation of one of the conformers in Figure 24 (yellow) covered with a layer of hydration water molecules (dotted blue spheres).

indicates that the dehydration of the polypeptide surface will hardly ever be a rate-limiting step either in protein folding or in intermolecular interactions with proteins.

The BPTI crystal structure contains four interior hydration water molecules. These are an integral part of the molecular architecture and are inaccessible to the solvent in a rigid model of the three-dimensional structure (Figure 26).

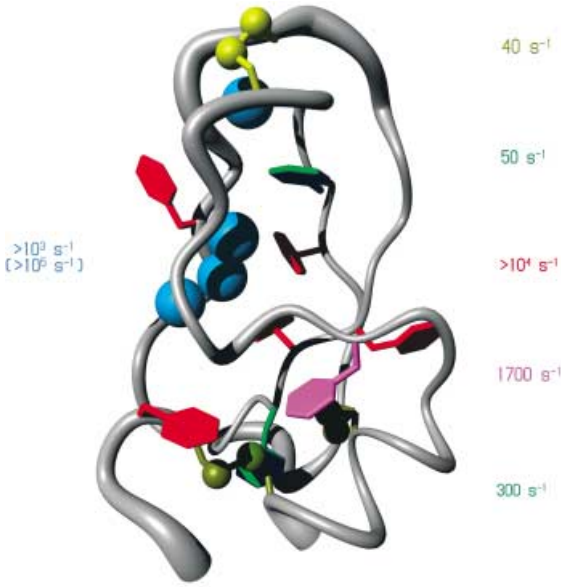


Figure 26. Intramolecular rate processes in BPTI. The polypeptide backbone is represented by a grey spline function through the α carbon positions, with the thickness of the line representing the spread of the bundle of 20 conformers in Figure 24. Individual rate processes and their frequencies are indicated with the following color code: green, magenta, and red: ring flips of phenylalanine and tyrosine; yellow: exchange between the *R* and *S* chiral forms of the disulfide bond at the top; cyan: interior hydration water molecules with indication of the exchange rates with the bulk water.

The chemical-shift dispersion anticipated for these four water molecules on the basis of the considerations in Figures 4–6 was not observed. This degeneracy of the chemical shifts of bound water and bulk water was found to be due to rapid exchange of water molecules in and out of the protein molecule, with an upper limit on the life-time of about 1 millisecond (see reference[53]; as indicated in Figure 26 by the number in parentheses, the actual life times for individual waters may be significantly shorter). Rapid exchange of interior hydration water molecules appears to be a general property of globular proteins, and was also observed for water molecules located at protein–DNA interfaces, for example, in the DNA complex with the *Antennapedia* homeodomain.

Another intriguing NMR observation of internal protein mobility are the 180° ring-flipping motions of phenylalanine and tyrosine.[54] Observation of these ring flips on the millisecond to microsecond timescale (Figure 26) was a genuine surprise for the following reasons: In the refined X-ray crystal structure of BPTI reported in 1975, the aromatic rings of phenylalanine and tyrosine are among the best-defined side chains, with the smallest temperature factors. In each ring the relative values of the temperature factors for the individual atoms increase toward the periphery, so that the largest positional uncertainty is indicated for the peripheral ζ-carbon atom on the symmetry axis through the C^{β}–C^{γ} bond, rather than for the four δ- and ε-ring carbon atoms, which undergo extensive movements during the ring flips. Theoretical studies then resolved this apparent contradiction: The crystallographic temperature factors sample multiple rotation states about the C^{α}–C^{β} bond, but they do not manifest the ring flips because the populations of all non-equilibrium rotational states about the C^{β}–C^{γ} bond are vanishingly small. Although the flipping motions about the C^{β}–C^{γ} bond have low frequencies (Figure 26), they are very rapid 180° rotations connecting two indistinguishable equilibrium orientations of the ring.

Similar to the exchange of internal hydration waters, the ring-flip phenomenon is a general feature of globular proteins, manifesting ubiquitous low-frequency internal motions that have activation energies of 60–100 kJ mol^{-1}, amplitudes larger than 1 Å, activation volumes of about 50 Å^3, and involve concerted displacement of numerous groups of atoms. Combined with sequence-specific NMR assignments, these experiments provide high spatio-temporal resolution for the description of rate processes in proteins. In Figure 26, this is illustrated by a mapping of the frequencies for ring flips and water exchange onto the NMR structure of BPTI.

In addition to the ring flips and the exchange of internal hydration water molecules, Figure 26 includes data on the exchange of a disulfide bond between the *R* and *S* chiral states. In contrast to the other two phenomena, this rate process connects two different molecular structures.

Although all the data collected in Figure 26 have been known for more than a decade, and some of them for nearly three decades, no widely accepted functional interpretation of these low-frequency motional processes has been advanced. The same holds for the conformational equilibria manifested by the protection factors governing the amide proton exchange rates in folded proteins (Figures 14 and 18). Quite possibly these NMR measurements are ahead of their times, and represent a source for future novel insights into structure–function correlations in proteins.

5. Outlook to NMR Applications in Structural and Functional Proteomics

With the availability of a rapidly increasing number of completely sequenced genomes, new challenges arise for the methods used for three-dimensional structure determination. On the one hand, "structural genomics" initiatives in several leading research centers focus on the development of technology for high-throughput structure determination to generate a comprehensive atlas of protein folds, so that remaining gaps could be filled by structure prediction methods. There is clearly a lot of room to further enhance the efficiency of each step of the NMR structure determination procedure (Table 1). On the other hand, we face the situation that newly determined protein structures should

enable us to predict novel functions, whereas in classical structural biology one encounters more typically the challenge of rationalizing known functions on the basis of the three-dimensional structure. This section describes some recent work in our laboratory that may eventually contribute to future strategies for the discovery of new physiological functions from molecular structure data.

It has been widely recognized that supplementing the determination of new protein folds with data on intermolecular interactions may provide a key for the identification of unknown gene functions. Since efficient use of conventional NMR spectroscopy in solution had been limited to particle sizes with molecular weights up to about 30000 Da (Figure 27, left), a new challenge for solution NMR techniques then arose from the fact that the supramolecular structures resulting from interactions of two or several proteins, or of other macromolecular components, tend to have high molecular weights. Although a 30000-Dalton size limit allowed work with a large pool of physiologically interesting proteins, it was hardly compatible with extensive use of NMR for studies of such supramolecular structures. For example, this size limit would severely narrow down the range of potential receptor systems accessible to NMR in drug discovery projects,[55,56] restrict studies of protein–nucleic acid complexes (Figure 2) to a small number of systems with modest size, and prevent the use of solution NMR for studies of membrane proteins, since these have to be reconstituted and solubilized in mixed micelles with surfactants or lipids (Figure 27, right).

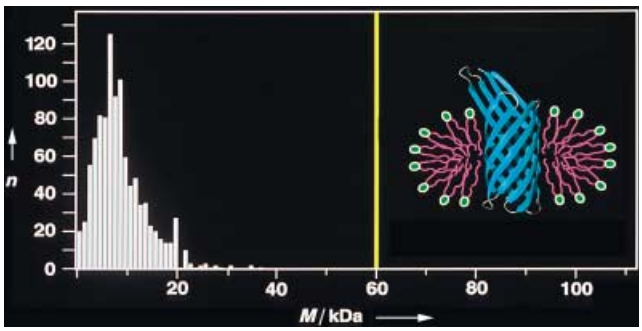


Figure 27. NMR structure determination and molecular weight. The horizontal axis covers the molecular weight range 0–110000 Da. The left side shows the molecular weight distribution of the NMR structures in the protein databank (December 2000). On the right, the structure of the protein OmpX from *E. coli* ($M \approx 18000$) in DHPC micelles ($M \approx 70000$ for the mixed micelles) indicates along the horizontal axis the approximate molecular weight range for NMR structure determination of mixed micelles with membrane proteins that is presently accessible with the NMR techniques of Figures 28–30.

A few years ago this limitation was successfully challenged, since the size range for applications of solution NMR techniques could be significantly extended through the introduction of transverse relaxation-optimized spectroscopy (TROSY).[57] As an illustration, Figure 28 shows a [^{15}N,^{1}H]-TROSY correlation spectrum of a membrane protein reconstituted in detergent micelles. Sharp, well-separated peaks are obtained in spite of the large size of the mixed micelles (Figure 27, right). With the use of the TROSY principle, the more complex NMR experiments needed for a structure determination with [^{2}H,^{13}C,^{15}N]-labeled proteins can also be obtained with high quality,[58] so that solution NMR can now be used for de novo membrane protein structure determination.[59]

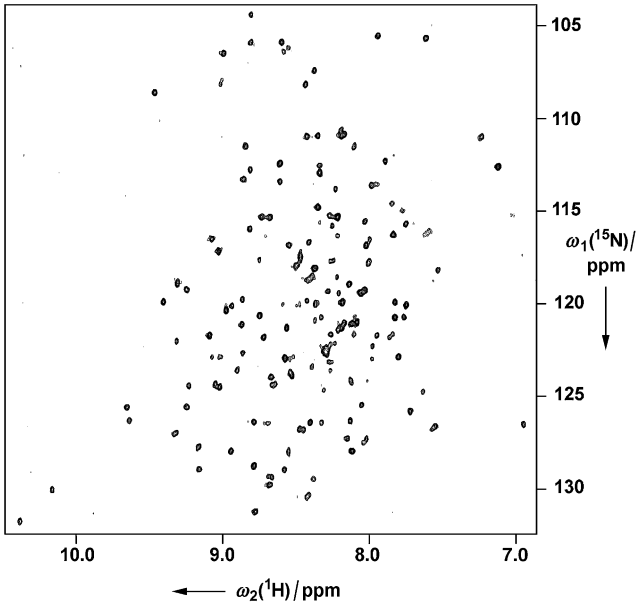


Figure 28. Transverse relaxation-optimized [^{15}N,^{1}H]-correlation spectroscopy (TROSY). A spectrum of the uniformly ^{2}H- and ^{15}N-labelled membrane protein OmpX reconstituted in DHPC micelles is shown (750 MHz, 30 °C, H_2O solution).

The improved quality of the NMR spectra of large structures with the use of TROSY can be qualitatively rationalized by the following considerations. As has previously been indicated in the discussion on NOEs [Section 3.1, Eq. (1)], the appearance of solution NMR spectra is intimately related to the effective correlation time, τ_c, which characterizes the thermal motions of the molecule considered. The increase of the τ_c values in larger structures results in line broadening due to rapid transverse spin relaxation. For example, if the spectrum of Figure 28 had been recorded with conventional NMR techniques, most of the resonance lines would not be individually resolved due to severe line broadening, and one would have experienced a severe loss of sensitivity for detection of the NMR signals. The reduced sensitivity can be readily appreciated by examination of the free induction decays (FID) in the time domain data. Figure 29 shows that rapid loss of magnetization in a conventional NMR experiment with a large structure can be slowed down by the use of TROSY, which corresponds to the reduction of the line width in the frequency domain spectrum.

The impact on the sensitivity is visualized in Figure 30 with a simplified scheme for a 2D correlation experiment, which includes two magnetization transfer periods, and the evolution and acquisition periods. During evolution and acquisition, the system is not subjected to external perturbations, and loss of magnetization occurs at a rate determined by the transverse relaxation time. It is readily apparent that rapid loss of magnetization in a conventional NMR experiment

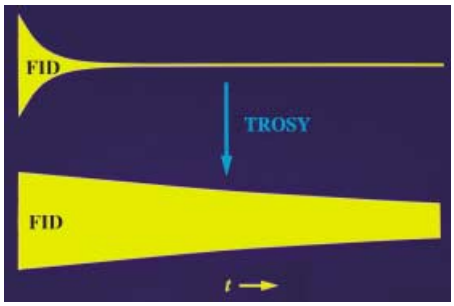


Figure 29. Schematic drawing of the free induction decay (FID) for a large protein with and without the use of TROSY. The period covered along the time axis is of the order of 100 ms. The FID represents the primary recording of the NMR data in the time domain, from which the frequency domain spectrum (for example, Figures 15, 16, and 28) is obtained by Fourier transformation.[31,33]

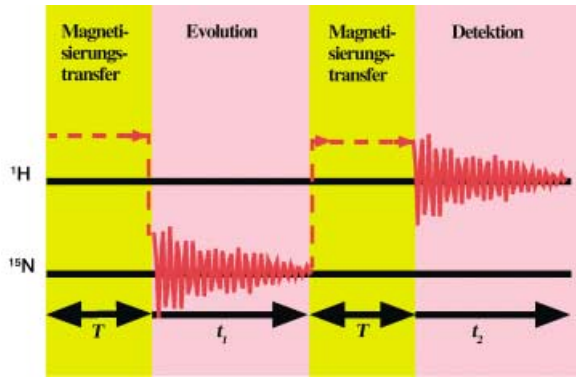


Figure 30. Basic features of a 2D [^{15}N,^{1}H]-correlation experiment. On the left, ^{1}H and ^{15}N indicate radio-frequency channels for irradiation of these isotopes. Yellow shading identifies the $^1H \rightarrow {}^{15}N$ and $^{15}N \rightarrow {}^1H$ magnetization transfer periods, T, and pink shading the evolution and acquisition periods, t_1 and t_2, respectively. During t_1 and t_2 the decay of the magnetization (FID) is schematically indicated. The overall duration of the experiment, $2T+t_1+t_2$, can be of the order of 10 to 1000 milliseconds, depending on the size of the structure studied and the intended purpose of the measurement.

(upper trace in Figure 29) leads to weak or even vanishing signal intensity at the end of the evolution period, and accordingly the sensitivity for detection of the signal during the acquisition period is very low. In contrast, one can obtain much improved sensitivity with the use of TROSY (lower trace in Figure 29), since plenty of signal intensity will be preserved at the end of the evolution period, and the signal can be recorded with high signal-to-noise ratio during a large portion of the acquisition period. Following such considerations for minimizing the loss of magnetization during all four time periods indicated in Figure 30, and with additional optimization of the magnetization transfer techniques,[60] solution NMR spectra have by now been recorded for structures with molecular weights up to 870000. In the spectrum of the co-chaperonin GroES bound to the chaperonin GroEL (Figure 31), resonance lines that provided novel information on structural and dynamic features of the GroES–GroEL interface are colored in red and blue.[61]

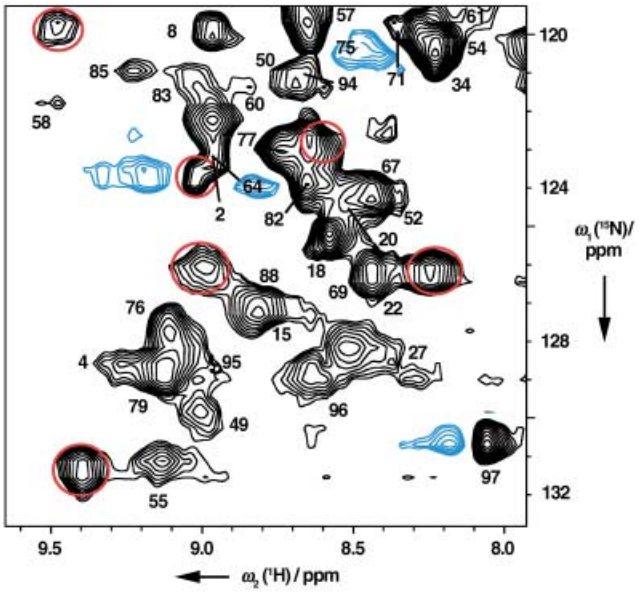


Figure 31. [^{15}N,^{1}H]-Correlation spectrum of the co-chaperonin GroES ($M \approx 70000$) bound to the chaperonin GroEL ($M \approx 800000$). The spectrum was recorded with a [^{15}N,^{1}H]-CRIPT-TROSY experiment.[60,61] The red and blue coloring is explained in the text.

The principal contributions to the rate of transverse spin-relaxation can be traced to two different types of interactions, that is, dipole–dipole coupling of the observed spin with other nearby spins and chemical-shift anisotropy (CSA).[28] These interactions are modulated by the stochastic rotational motions in solution, and as a consequence the rate of transverse relaxation increases for larger structures with slower Brownian motions. TROSY exploits constructive interference between the two relaxation mechanisms, and actually uses CSA relaxation at high fields to cancel the dipolar relaxation.[57] In this way the appearance of the NMR spectrum is effectively uncoupled from the Brownian motions, which then enables the recording of solution NMR spectra with large structures.

It remains to be seen how these new NMR techniques will be employed most profitably in the future. Intriguing possibilities include that NMR can now be employed in drug discovery projects with very large receptors.[55,56] Combined with suitable isotope-labelling strategies, TROSY-based NMR techniques have also been shown to provide a powerful approach for investigations of intermolecular interactions in supramolecular structures with two or several macromolecular components.[62,63] In these applications, a detailed structural interpretation of the NMR data will in most instances be dependent on the availability of an independently determined atomic-resolution structure for one or multiple components, which may have been obtained either by NMR in solution or by diffraction methods in single crystals. Applications of the new NMR techniques for de novo determination of large structures appears to be particularly attractive for, but not limited to, nucleic acid–protein complexes and small membrane proteins reconstituted in soluble detergent or lipid micelles.

The characterization of a membrane protein structure has also been extended to include the detergents in the mixed micelles (Figure 32),[64] since the areas of the protein surface in contact with the detergent molecules could be delineated

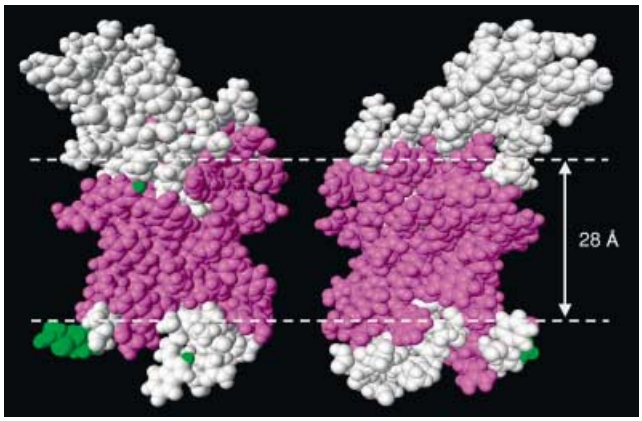


Figure 32. NMR structure of the membrane protein OmpX reconstituted in DHPC micelles. Front and back views of a space-filling model of OmpX are shown, with pink and green coloring identifying the surface area of the protein that is in contact with DHPC molecules in the mixed micelles. The two broken horizontal lines indicate the thickness of the lipid phase in the *E. coli* cell membrane.

by the observation of intermolecular NOEs between hydrogen atoms of the protein and the detergent. In these particular mixed micelles this surface area is virtually identical to the protein surface in contact with the lipid phase in the biological membrane. Since this leaves both ends of the β barrel of OmpX freely accessible to the aqueous solvent, this reconstitution system should also be suitable for functional studies of the membrane protein with NMR.

This last section of my presentation certainly indicates only a narrow range of potential further developments of solution NMR techniques and their applications in structural biology and structural proteomics. I look forward with great expectations to the future evolution of this awesome and beautiful technique, which has given me so many years of joy and excitement in studies of the molecules of life.

From 1970 through 2002, 229 students, postdoctoral research associates, and technical and administrative staff worked with me at the ETH Zürich. I am deeply indebted to all of them for their enthusiasm and dedication, which resulted in the work summarized in this article. Their names and individual contributions can be found in the reference list. Although human minds stand behind all progress in science, the success of our research projects also depended critically on financial resources. I would like to acknowledge the ETH Zürich, the Swiss National Science Foundation, the Kommission für Technologie und Innovation (KTI), Bruker-Biospin AG, and the Scripps Research Institute in La Jolla, CA, USA, for their support.

Received: March 19, 2003 [A595]

[1] "The Structure of the *Antennapedia* Homeodomain Determined by NMR Spectroscopy in Solution: Comparison with Prokaryotic Repressors": Y. Q. Qian, M. Billeter, G. Otting, M. Müller, W. J. Gehring, K. Wüthrich, *Cell* **1989**, *59*, 573–580.
[2] "Protein–DNA Contacts in the Structure of a Homeodomain–DNA Complex Determined by Nuclear Magnetic Resonance Spectroscopy in Solution": G. Otting, Y. Q. Qian, M. Billeter, M. Müller, M. Affolter, W. J. Gehring, K. Wüthrich, *EMBO J.* **1990**, *9*, 3085–3092.
[3] "NMR Structure of the Bovine Prion Protein": F. Lopez Garcia, R. Zahn, R. Riek, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8334–8399.
[4] K. Wüthrich, *NMR of Proteins and Nucleic Acids*, Wiley, New York, **1986**.
[5] "NMR—This Other Method for Protein and Nucleic Acid Structure Determination": K. Wüthrich, *Acta Crystallogr. Sect. D* **1995**, *51*, 249–270.
[6] K. Wüthrich, *NMR in Structural Biology: A Collection of Papers by Kurt Wüthrich*, World Scientific, Singapore, **1995**.
[7] "The Way to NMR Structures of Proteins": K. Wüthrich, *Nat. Struct. Biol* **2001**, *8*, 923–925.
[8] "Nuclear Magnetic Resonance Spectra of Proteins": M. Saunders, A. Wishnia, *Ann. N. Y. Acad. Sci.* **1958**, *70*, 870–874.
[9] "Proton Magnetic Resonance Spectra of Amino Acids": O. Jardetzky, C. D. Jardetzky, *J. Biol. Chem.* **1958**, *233*, 383–387.
[10] "Nuclear Magnetic Resonance Studies of Proteins": A. Kowalsky, *J. Biol. Chem.* **1962**, *237*, 1807–1819.
[11] "Proton Magnetic Resonance Spectra of Some Proteins": M. Mandel, *J. Biol. Chem.* **1965**, *240*, 1586–1592.
[12] "Manifestations of the Tertiary Structures of Proteins in High-Frequency Nuclear Magnetic Resonance": C. C. McDonald, W. D. Phillips, *J. Am. Chem. Soc.* **1967**, *89*, 6332–6341.
[13] "High Resolution Proton Nuclear Magnetic Resonance Spectroscopy of Cytochrome *c*": K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1969**, *63*, 1071–1078.
[14] "The Absence of "Heme–Heme" Interactions in Hemoglobin": R. G. Shulman, S. Ogawa, K. Wüthrich, T. Yamane, J. Peisach, W. E. Blumberg, *Science* **1969**, *165*, 251–257.
[15] "NMR Studies of Hemoglobins VI: Heme Proton Spectra of Human Deoxyhemoglobins and Their Relevance to the Nature of Co-operative Oxygenation of Haemoglobin": D. G. Davis, T. R. Lindstrom, N. H. Mock, J. J. Baldassare, S. Charache, R. T. Jones, C. Ho, *J. Mol. Biol.* **1971**, *60*, 101–111.
[16] "Paramagnetic Proton NMR Shifts of Metmyoglobin, Methemoglobin, and Hemin Derivatives": R. J. Kurland, D. G. Davis, C. Ho, *J. Am. Chem. Soc.* **1968**, *90*, 2700–2701.
[17] "Structural Studies of Hemes and Hemoproteins by Nuclear Magnetic Resonance Spectroscopy": K. Wüthrich, *Struct. Bonding (Berlin)* **1970**, *8*, 53–121.
[18] "Electronic Structure of Cyanide Complexes of Hemes and Heme Proteins": R. G. Shulman, S. H. Glarum, M. Karplus, *J. Mol. Biol.* **1971**, *57*, 93–115.
[19] K. Wüthrich, *NMR in Biological Research: Peptides and Proteins*, North Holland, Amsterdam, **1976**.
[20] J. H. Noggle, R. E. Schirmer, *The Nuclear Overhauser Effect*, Academic Press, New York, **1971**.
[21] "Homonuclear INDOR Spectroscopy as a Means of Simplifying and Analyzing Proton Magnetic Resonance Spectra of Peptides and as a Basis for Determining Secondary and Tertiary Conformations of Complex Peptides": W. A. Gibbons, H. Alms, R. S. Bockman, H. R. Wyssbrod, *Biochemistry* **1972**, *11*, 1721–1725.
[22] "Double Resonance NMR Observation of Electron Exchange Between Ferri- and Ferrocytochrome *c*": R. K. Gupta, A. G. Redfield, *Science* **1970**, *169*, 1204–1206.

[23] "Negative Nuclear Overhauser Effects as Probes of Macromolecular Structure": P. Balaram, A. A. Bothner-By, J. Dadok, *J. Am. Chem. Soc.* **1972**, *94*, 4015 – 4017.
[24] "Relaxation Processes in a System of Two Spins": I. Solomon, *Phys. Rev.* **1955**, *99*, 559 – 565..
[25] "Proton Magnetic Relaxation and Spin Diffusion in Proteins": A. Kalk, H. J. C. Berendsen, *J. Magn. Reson.* **1976**, *24*, 343 – 366.
[26] "Transient Proton–Proton Overhauser Effects in Horse Ferrocytochrome *c*": S. L. Gordon, K. Wüthrich, *J. Am. Chem. Soc.* **1978**, *100*, 7094 – 7096.
[27] "Truncated Driven Nuclear Overhauser Effect (TOE): a New Technique for Studies of Selective ^{1}H–^{1}H Overhauser Effects in the Presence of Spin Diffusion": G. Wagner, K. Wüthrich, *J. Magn. Reson.* **1979**, *33*, 675 – 680.
[28] A. Abragam, *Principles of Nuclear Magnetism*, Clarendon Press, Oxford, **1961**.
[29] "Evolutionary Change of the Heme *c* Electronic Structure: Ferricytochrome *c*-551 from *Pseudomonas Aeruginosa* and Horse Heart Ferricytochrome *c*": R. M. Keller, K. Wüthrich, *Biochem. Biophys. Res. Commun.* **1978**, *83*, 1132 – 1139.
[30] "Individual Assignments of Amide Proton Resonances in the Proton NMR Spectrum of the Basic Pancreatic Trypsin Inhibitor": A. Dubs, G. Wagner, K. Wüthrich, *Biochim. Biophys. Acta* **1979**, *577*, 177 – 194.
[31] "Nuclear Magnetic Resonance Fourier Transform Spectroscopy": R. R. Ernst, *Angew. Chem.* **1992**, *104*, 817 – 836; *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 805 – 823.
[32] "Homonuclear Two-Dimensional ^{1}H NMR of Proteins: Experimental Procedures": G. Wider, S. Macura, A. Kumar, R. R. Ernst, K. Wüthrich, *J. Magn. Reson.* **1984**, *56*, 207 – 234.
[33] R. R. Ernst, G. Bodenhausen, A. Wokaun, *Principles of Nuclear Magnetic Resonance in One and Two Dimensions*, Oxford University Press, Oxford, **1987**.
[34] "Sequential Resonance Assignments in Protein ^{1}H Nuclear Magnetic Resonance Spectra: Basic Pancreatic Trypsin Inhibitor": G. Wagner, K. Wüthrich, *J. Mol. Biol.* **1982**, *155*, 347 – 366.
[35] "Systematic Application of Two-Dimensional ^{1}H Nuclear Magnetic Resonance Techniques for Studies of Proteins 2: Combined Use of Correlated Spectroscopy and Nuclear Overhauser Spectroscopy for Sequential Assignments of Backbone Resonances and Elucidation of Polypeptide Secondary Structures": G. Wagner, Anil-Kumar, K. Wüthrich, *Eur. J. Biochem.* **1981**, *114*, 375 – 384.
[36] "Sequential Resonance Assignments in Protein ^{1}H Nuclear Magnetic Resonance Spectra: Computation of Sterically Allowed Proton–Proton Distances and Statistical Analysis of Proton–Proton Distances in Single Crystal Protein Conformations": M. Billeter, W. Braun, K. Wüthrich, *J. Mol. Biol.* **1982**, *155*, 321 – 346.
[37] "Polypeptide Secondary Structure Determination by Nuclear Magnetic Resonance Observation of Short Proton–Proton Distances": K. Wüthrich, M. Billeter, W. Braun, *J. Mol. Biol.* **1984**, *180*, 715 – 740.
[38] "Calibration of the Angular Dependence of the Amide Proton–C^α Proton Coupling Constants, $^3J_{HN\alpha}$, in a Globular Protein: Use of $^3J_{HN\alpha}$ for Identification of Helical Secondary Structure": A. Pardi, M. Billeter, K. Wüthrich, *J. Mol. Biol.* **1984**, *180*, 741 – 751.
[39] "Sequential Resonance Assignments as a Basis for Determination of Spatial Protein Structures by High Resolution Proton Nuclear Magnetic Resonance": K. Wüthrich, G. Wider, G. Wagner, W. Braun, *J. Mol. Biol.* **1982**, *155*, 311 – 319.
[40] "Combined Use of Proton–Proton Overhauser Enhancements and a Distance Geometry Algorithm for Determination of Polypeptide Conformations: Application to Micelle-Bound Glucagon": W. Braun, C. Bösch, L. R. Brown, N. Gō, K. Wüthrich, *Biochim. Biophys. Acta* **1981**, *667*, 377 – 396.
[41] "A Distance Geometry Program for Determining the Structures of Small Proteins and Other Macromolecules from Nuclear Magnetic Resonance Measurements of Intramolecular ^{1}H–^{1}H Proximities in Solution": T. F. Havel, K. Wüthrich, *Bull. Math. Biol.* **1984**, *46*, 673 – 698.
[42] "An Evaluation of the Combined Use of Nuclear Magnetic Resonance and Distance Geometry for the Determination of Protein Conformations in Solution": T. F. Havel, K. Wüthrich, *J. Mol. Biol.* **1985**, *182*, 281 – 294.
[43] "Solution Conformation of Proteinase Inhibitor IIA from Bull Seminal Plasma by ^{1}H Nuclear Magnetic Resonance and Distance Geometry": M. P. Williamson, T. F. Havel, K. Wüthrich, *J. Mol. Biol.* **1985**, *182*, 295 – 315.
[44] "Prospects for NMR of Large Proteins": G. Wagner, *J. Biomol. NMR* **1993**, *3*, 375 – 385.
[45] "Solution NMR Spectroscopy Beyond 25 kDa": L. E. Kay, K. H. Gardner, *Curr. Opin. Struct. Biol.* **1997**, *7*, 564 – 570.
[46] "Methodological Advances in Protein NMR": A. Bax, S. Grzesiek, *Acc. Chem. Res.* **1993**, *26*, 131 – 138.
[47] "Technical Aspects of NMR Spectroscopy with Biological Macromolecules and Studies of Hydration in Solution": G. Wider, *Prog. Nucl. Magn. Reson. Spectrosc.* **1998**, *32*, 193 – 275.
[48] "Heteronuclear Filters in Two-Dimensional [^{1}H,^{1}H]-NMR Spectroscopy: Combined Use with Isotope Labelling for Studies of Macromolecular Conformation and Intermolecular Interactions": G. Otting, K. Wüthrich, *Q. Rev. Biophys.* **1990**, *23*, 39 – 96.
[49] "Structure Calculation of Biological Macromolecules from NMR Data": P. Güntert, *Q. Rev. Biophys.* **1998**, *31*, 145 – 237. .
[50] "Automated Analysis of NMR Assignments and Structures for Proteins": H. N. B. Moseley, G. T. Montelione, *Curr. Opin. Struct. Biol.* **1999**, *9*, 635 – 642.
[51] "Protein NMR Structure Determination with Automated NOE Identification in the NOESY Spectra Using the New Software ATNOS": T. Herrmann, P. Güntert, K. Wüthrich, *J. Biomol. NMR* **2002**, *24*, 171 – 189.
[52] "Semi-Classical Nuclear Spin Relaxation Theory Revisited for Use with Biological Macromolecules": P. Luginbühl, K. Wüthrich, *Prog. Nucl. Magn. Reson. Spectr.* **2002**, *40*, 199 – 247.
[53] "Protein Hydration in Aqueous Solution": G. Otting, E. Liepinsh, K. Wüthrich, *Science* **1991**, *254*, 974 – 980.
[54] "NMR Investigations of the Dynamics of the Aromatic Amino Acid Residues in the Basic Pancreatic Trypsin Inhibitor": K. Wüthrich, G. Wagner, *FEBS Lett.* **1975**, *50*, 265 – 268.
[55] "Discovering High-Affinity Ligands for Proteins: SAR by NMR": S. B. Shuker, P. J. Hajduk, R. P. Meadows, S. W. Fesik, *Science* **1996**, *274*, 1531 – 1534.
[56] "NMR in Drug Discovery": M. Pellecchia, D. S. Sem, K. Wüthrich, *Nat. Rev. Drug Discovery* **2002**, *1*, 211 – 219.
[57] "Attenuated T_2 Relaxation by Mutual Cancellation of Dipole–Dipole Coupling and Chemical Shift Anisotropy Indicates an Avenue to NMR Structures of Very Large Biological Macromolecules in Solution": K. Pervushin, R. Riek, G. Wider, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12366 – 12371.
[58] "TROSY in Triple-Resonance Experiments: New Perspectives for Sequential NMR Assignment of Large Proteins": M. Salzmann, K. Pervushin, G. Wider, H. Senn, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 13585 – 13590.
[59] "Solution NMR Studies of the Integral Membrane Proteins OmpX and OmpA from *Escherichia coli*": C. Fernández, C. Hilty, S. Bonjour, K. Adeishvili, K. Pervushin, K. Wüthrich, *FEBS Lett.* **2001**, *504*, 173 – 178.
[60] "Polarization Transfer by Cross-Correlated Relaxation in Solution NMR with Very Large Molecules": R. Riek, G. Wider, K. Pervushin, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4918 – 4923.
[61] "NMR Analysis of a 900 K GroEL–GroES Complex": J. Fiaux, E. Bertelsen, A. Horwich, K. Wüthrich, *Nature* **2002**, *418*, 207 – 211.

[62] "Pilus Chaperone FimC–Adhesin FimH Interactions Mapped by TROSY-NMR": M. Pellecchia, P. Sebbel, U. Hermanns, K. Wüthrich, R. Glockshuber, *Nat. Struct. Biol.* **1999**, *6*, 336–339.
[63] "TROSY-NMR Reveals Interaction between ERp57 and the Tip of the Calreticulin P-Domain": E. M. Frickel, R. Riek, I. Jelesarov, A. Helenius, K. Wüthrich, L. Ellgaard, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1954–1959.
[64] "Lipid–Protein Interactions in DHPC Micelles Containing the Integral Membrane Protein OmpX Investigated by NMR Spectroscopy": C. Fernández, C. Hilty, G. Wider, K. Wüthrich, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 13533–13537.

