Brownian motion, spin diffusion and protein structure determination in solution

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Perspectives in Magnetic Resonance

Brownian motion, spin diffusion and protein structure determination in solution

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ABSTRACT

This paper presents my recollections on the development of protein structure determination by NMR in solution from 1968 to 1992. The key to success was to identify NMR-accessible parameters that unambiguously determine the spatial arrangement of polypeptide chains. Inspired by work with cyclopeptides, model considerations showed that enforcing short non-bonding interatomic distances imposes «ring closure conditions» on polypeptide chains. Given that distances are scalar parameters, this indicated an avenue for studies of proteins in solution, i.e., under the regime of stochastic rotational and translational motions at frequencies in the nanosecond range (Brownian motion), where sharp pictures could not be obtained by photography-related methods. Later-on, we used distance geometry calculations with sets of inter-atomic distances derived from protein crystal structures to confirm that measurements of short proton—proton distances could provide atomic-resolution structures of globular proteins. During the years 1976–1984 the following four lines of research then led to protein structure determination by NMR in solution. First, the development of NMR experiments enabling the use of the nuclear Overhauser effect (NOE) for measurements of interatomic distances between pairs of hydrogen atoms in proteins. Second, obtaining sequence-specific resonance assignment solved the "phase problem" for protein structure determination by NMR. Third, generating and programming novel distance geometry algorithms enabled the calculation of atomic-resolution protein structures from limited sets of distance constraints measured by NMR. Fourth, the introduction of two-dimensional NMR provided greatly improved spectral resolution of the complex spectra of proteins as well as efficient delineation of scalar and dipole–dipole ^{1}H–^{1}H connectivities, thus making protein structure determination in solution viable and attractive.

1. Introduction

"för hans utveckling av kärnmagnetisk resonansspektroskopi för bestämming av biologiska makromolekylers tredimensionella struktur i lösning" (official translation: *"for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution*"); this is the citation in the Nobel Prize diploma that was handed to me on December 10, 2002. The present paper describes my recollections of key events on the way to atomic-resolution protein structure determination by NMR. Equally important NMR investigations of conformational polymorphisms and rate processes in biological systems, which complement structure determinations by NMR, X-ray crystallography and cryo-electron microscopy (cryo-EM), may be the focus of another historical account, which would cover seminal contributions by distinguished members of the NMR community that date back more than two decades before the first structure determination of a globular protein by NMR in 1984 [1].

In the early days of NMR spectroscopy with biological macromolecules, from 1957 into the 1980s, only a few proteins were accessible for structural studies, either by X-ray crystallography or by NMR in solution, mainly because milligram quantities of the proteins had to be isolated and purified from natural sources. NMR spectroscopists were therefore bound to work mainly with those few proteins that were also subject to structure determination by X-ray crystallography. As a consequence, the global polypeptide folds in the protein crystal structures were extensively used to support the interpretation of NMR data recorded with the same proteins (for example, [2–4]). Limitations of then-available NMR techniques for structural studies of biological macromolecules were therefore often overlooked. I fully realized this situation in 1975 when writing a monograph, *NMR in Biological Research: Peptides and Proteins* [5]. From 1976 onward my group therefore re-oriented the search for a method of *de novo protein* structure determination by NMR in solution, on the one hand toward investigations of the spin physics under the regime of

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Brownian motion, and on the other hand to building molecular models along the lines that had contributed to success with protein structure determination by X-ray crystallography. Important guidance was obtained from discussions with Professor Jack Dunitz at the ETH Zürich [6], and from publications by Linus Pauling on polypeptide secondary structures [7] and by G.N. Ramachandran on sterically allowed local polypeptide conformations [8]. Throughout, we were in close contact with colleagues working in protein crystallography, in particular with Max Perutz, Robert Huber, Hans Deisenhofer, David Philips, Fred Richards, Tom Blundell, and Jane and David Richardson. Protein crystallography was years ahead of NMR for structural studies of biological macromolecules, and we benefited in key issues from their experience, in particular from the early efforts to refine [9] and classify [10] protein crystal structures.

2. Background from high school and universities

My education, leading to a Ph.D. degree, was pursued in Switzerland, which is my home country. I went to the Swiss version of "high school" at the Gymnasium Biel/Bienne from 1952 to 1957. Biel/Bienne is a bilingual city located at the outlet of a lake ("Bielersee"/"Lac de Bienne") at the foot of the Jura mountains. In my specialization, with a focus on mathematics, physics and chemistry, we were a small class of seven students, and our teachers were ambitious to change from the employment as teachers at the Gymnasium to a Chair in one of the Swiss Universities. The Gymnasium Biel/Bienne was closely attached to the Swiss Federal School of Sports in nearby Magglingen/Macolin, where we had free access to train in the company of the Swiss top athletes. During the Soccer World Cup 1954 we even enjoyed the presence of the Brazilian and Swiss National Teams, which had chosen to be accommodated in the sports school. Overall, a high level of teaching in the natural sciences was an ideal preparation for my university studies, and the Gymnasium education also instilled in me a love of the French language and culture, and a keen interest in competitive sports.

At the University of Bern I obtained a "Licentiat" degree in physics after two years; this was followed by "Licentiat" degrees in mathematics in 1960 and in chemistry in 1962. Obtaining my degrees early made it possible to accept part-time teaching jobs already from 1959 to 1962, first teaching physics at the "Kantonsschule Solothurn" for one year, and then teaching chemistry in Biel/Bienne for two years. I still feel that teaching the subjects of my studies benefited me more than it did the high school students who listened to me. After the primary studies in chemistry, physics and mathematics at the University of Bern, I obtained a Ph.D. degree in inorganic chemistry and a degree in sports education ("Eidgenössisches Turn- und Sport-lehrerdiplom") at the University of Basel. This unusual combination of graduate studies resulted from my slow way of deciding on what I really wanted to do with my life. My main interest was in sports, but I had by now realized that I was not sufficiently talented for success in high-level competition. I therefore changed my focus to sports education, with the prospect of a career as a high-school teacher in physical education and natural sciences. At the University of Basel, I started to follow the curriculum in sports education in the Spring of 1962; this included 25 to 30 h per week of personal training in a broad range of different sports, and lecture courses in human anatomy, physiology and hygiene at the medical school. By the Fall of 1962 I more and more felt an urge to include scientific research into my days. This required to choose between chemistry and physics, and between Basel and Bern. It all ended in a fortunate compromise when Professor Silvio Fallab accepted me as a graduate student in the Institute of Inorganic Chemistry at the University of Basel. Since this was not a paid employment and I actually paid a tuition fee, I enjoyed complete freedom to share my time between the sports studies and the Ph.D. research. With great generosity, Professor Fallab also arranged that I could pursue most of my thesis work in the Physics Institute at the University of Basel, and I could thus continue to share the time among my multiple areas of interest. In February 1964 I obtained the Ph.D. degree and the degree in sports education. I then spent an additional year in Basel, part-time teaching gymnastics at the women's Gymnasium "Am Kohlenberg", and part-time as a postdoctoral fellow with Professor Fallab.

For the JMR community, my anecdotal way to magnetic resonance spectroscopy may be of interest. Within a few weeks after I joined the Fallab group at the Institute of Inorganic Chemistry, a junior faculty, Peter Hemmerich [11], organized an international workshop on "Modern Methods of Structural Chemistry". Among the speakers were Anders Ehrenberg, Bo Malmström and Tore Vänngard, who later-on organized the second International Conference on Magnetic Resonance in Biological Systems (ICMRBS) in Stockholm and published a highly influential Proceedings volume from this meeting [12]. At the Basel workshop they presented their discovery of "blue copper proteins", which they analysed primarily with electron paramagnetic resonance (EPR) spectroscopy [12]. I was intrigued by these findings and inquired about the possibility of including EPR experiments into my thesis work. Professor Fallab was informed that there was an EPR spectrometer installed in the Physics Institute across the street from his Institute. This expensive instrument had been purchased for a young star in the Physics Institute, Dr. Peter Diehl [13], and its use was shared with the Physics Division of the Ciba Company under the direction of Professor Heinrich Labhardt [14]. At the time of my inquiry, the instrument in the Physics Institute was standing idle, because Peter Diehl was on sabbatical leave and the Ciba Company had purchased their own EPR spectrometer. After a few days of intense training by a member of the Ciba Physics Department, Dr. Hans Loeliger [15], and with only minimal theoretical background, I started my magnetic resonance career. As I had full-time access to the Varian X-band EPR spectrometer, I soon collected a wealth of data in support of investigations on redox reactions with copper ions [15]; this included that I looked into the secrets behind the blue copper proteins [16]. Motivated by a literature search, I also started studies of vanadyl complexes in solution, which present beautiful hyperfine structures [17].

As a postdoctoral fellow with Professor Robert E. (Bob) Connick at the University of California Berkeley, I combined EPR studies of vanadyl complexes with measurements of the hydration dynamics, using hydrogen, deuterium and oxygen-17 NMR [18,19], and we also studied very rapid water exchange in and out of the coordination sites on alkaline metal ions [20]. In addition to pursuing these experimental projects, I greatly benefited from the UC Berkeley environment to broaden my background education. I acquired a solid grasp of the theory of 3d-metal ions from working through the authoritative monograph by John Griffith [21] in the company of Alex von Zelewsky [22], a Swiss postdoctoral fellow with Bob Connick whose stay in Berkeley overlapped with mine. A broad basis in the theory of magnetic resonance resulted from intense work with the textbooks by Anatole Abragam [23] and Charles Slichter [24] in the "Connick Seminars". I also acquired hands-on training in group theory and quantum mechanics by a heavy involvement with the homework for a physics graduate course by Professor Michael Tinkham, where we tested the problems section of his famous monograph on the subject [25]. When I started NMR studies of proteins at the Bell Telephone Laboratories in 1967, I thus had a solid background in quantum mechanics and group theory, and five years of hands-on experience with NMR- and EPR-spectrometers. In addition, from my sports activities I had a keen interest in oxygen uptake by the human organism, which turned out to provide important guidance for the selection of my initial research projects with proteins.

3. From metal ions to polypeptide chains

At the Bell Telephone Laboratories in Murray Hill, NJ ("Bell Labs"), I was hired by Dr. Robert G. (Bob) Shulman to use my inorganic chemistry background for investigations of the physical–chemical properties of protein-bound metal ions. One of the envisaged projects was to bind vanadyl ions into the active site of carboxypeptidase A, and to compare the interactions with hydration water and other small ligands in this macromolecular environment with my earlier studies of vanadyl complexes with small-molecule ligands [17–19]. This project did not go very far, because vanadyl ions could not compete with the natural zinc ion in the carboxypeptidase A active site. Another project was started with a focus on function-related electronic states of the iron in myoglobin, hemoglobin and cytochrome *c*. These hemoproteins were among the less than ten proteins for which crystal structures were available in 1967. Because of my interest and practical experience with competitive sports, I was primarily interested in the oxygen uptake by hemoglobin and myoglobin; however, it was work with cytochromes *c* that had an important role in steering me toward the development of protein structure determination by NMR.

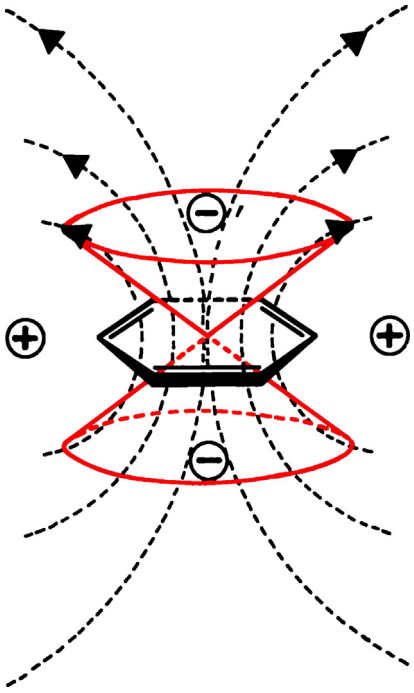

Fig. 1. Local "ring current field" around aromatic rings in solution induced by an external, static magnetic field. The shape of the ring current field is indicated by the red double-cone and by broken magnetic-field lines. The minus-sign indicates that the NMR lines of hydrogen atoms located inside the cone in the three-dimensional protein structure are shifted "upfield", and the plus sign indicates for atoms outside of the cone that the shifts are "downfield" (Figure taken from [26] with permission).

When I started to work with cytochrome *c*, my interest in polypeptide chains was limited to their providing ligands for metal ion complexation. Because of the fast electron spin relaxation of low spin ferric heme iron, ferricytochrome c shows a ^{1}H NMR spectrum with quite narrow paramagnetically shifted lines outside of the chemical shift range for diamagnetic compounds [4]. After I added potassium cyanide to ferricytochrome c solutions, these signals were at different chemical shifts, showing that the cyanide ion competed successfully with at least one of the natural iron ligands. I thus obtained the ferricytochrome c complex with cyanide at nearly 100% occupancy. In contrast, cyanide addition to ferrocytochrome c had no effect on the ^{1}H NMR spectrum, showing that the natural ligands in the ring current field (Fig. 1) of the heme group were not displaced by cyanide. In a redox reaction leading from the ferric to the ferrous state of the cyanide complex of cytochrome *c* (Fig. 2), I then observed a slow reaction that caused the appearance of new high-field resonance lines, which included a methyl resonance (Fig. 3); these had to originate from a natural ligand of the heme iron that had been replaced by cyanide in the

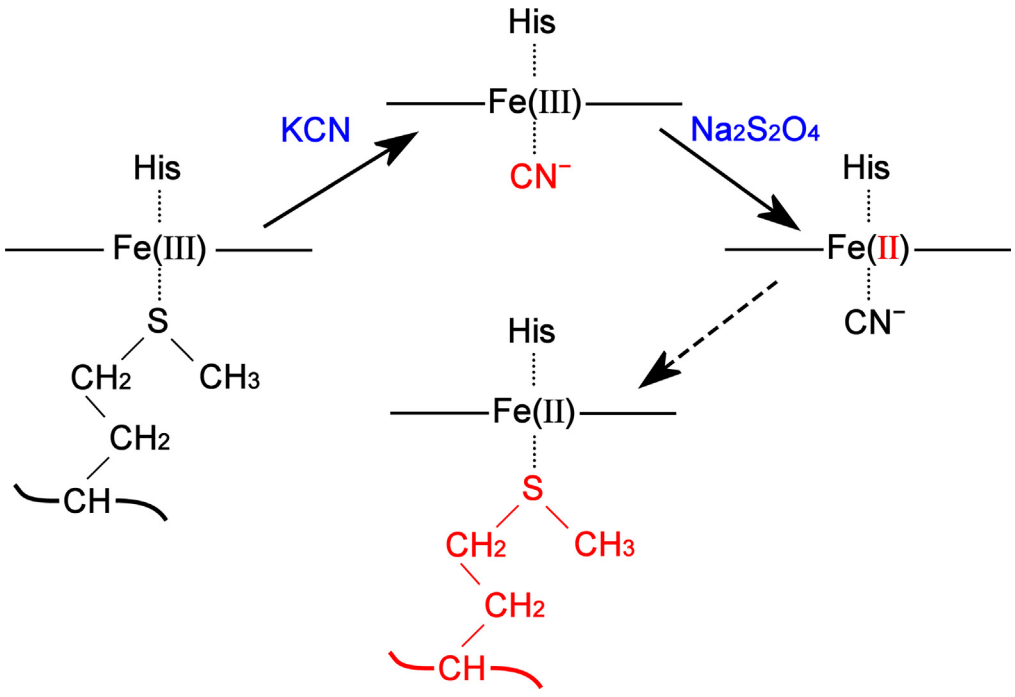

Fig. 2. Three chemical reaction steps with cytochrome *c* that were used to identify methionine as one of the axial ligands of the heme iron [4]. The Roman numerals indicate the oxidation state of the heme iron, and His stands for the amino acid histidine. The solid and broken arrows indicate rapid and slow reaction steps, respectively. Colour code: black, heme iron with ligands, where the horizontal line through Fe represents a side view of the heme c ring (see Fig. 6 below); blue, chemicals added to the protein solution; red, new features resulting from the addition of these chemicals (Figure taken from [26] with permission).

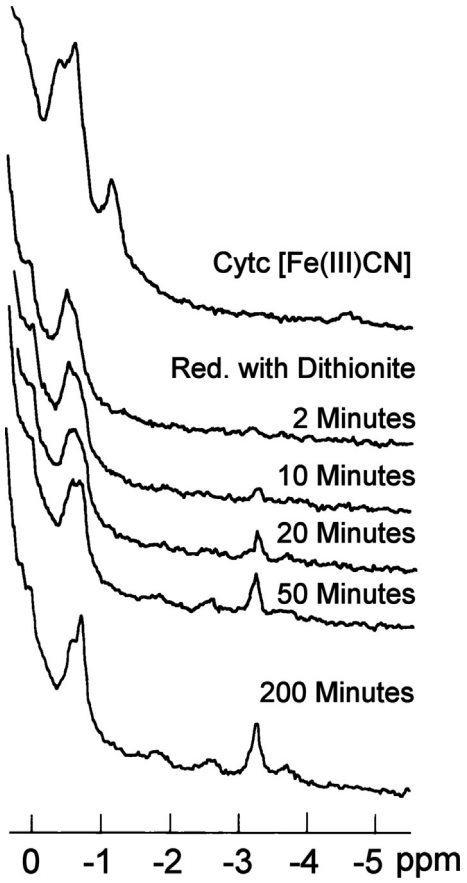

Fig. 3. Changes with time in the high-field region of the 1D ^{1}H NMR spectrum of guanaco cytochrome *c* after reducing the ferric cyanide complex (top trace) with dithionite (220 MHz, 9 °C, solvent $^{2}H_2O$) (Figure taken from [26] with permission).

ferric state (Figs. 2 and 3). From a textbook search for an amino acid that would bind to the heme iron and contain a methyl group, methione was unambiguously identified as an axial ligand of the heme iron (Fig. 2; on this occasion I also learned my lesson and memorized the chemical structures of the 20 proteinogenic amino acids). Inspection of the crystal structure then showed that whereas an axially coordinated histidine (Fig. 2) was unambiguously identified, identification of the second axial ligand was beyond the resolution of 1968 X-ray crystallography; this ligand had initially been identified as a lysine side chain and subsequently been left unassigned [27]. It was at this moment that I got convinced that NMR could provide *de novo* protein structure determination in solution, possibly even at higher resolution than X-ray crystallography.

For the hemoprotein projects at Bell Labs it was crucial that we were equipped with one of the first superconducting NMR spectrometers, a Varian-220 continuous wave (CW) instrument. When I joined the ETH Zürich in Switzerland in 1969, a similar spectrometer was available. I continued to work with hemoproteins, but also pursued NMR studies of oligopeptides and a very stable small protein, the basic pancreatic trypsin inhibitor (BPTI). While we made exciting observations on protein dynamics, there was little progress toward protein three-dimensional structure determination. A survey of the early literature on NMR studies with proteins [5] then convinced me that none of the approaches tried up to 1975 could lead to *de novo* protein structure determination. The early results obtained with cytochrome *c* had misled me to believe that *de novo* structure determination of proteins could be based on the variation of local micro-susceptibilities across globular proteins, in particular those caused by ring current fields near aromatic rings (Fig. 1) and by pseudo-contact shifts near paramagnetic ions. The 1975 literature search then opened my eyes for having overlooked the inherent ambiguities arising from the high symmetries of ring current fields (Fig. 1), which had routinely been resolved by inspection of the crystal structures of the proteins that we worked with.

4. With NMR to structures of proteins in solution

4.1. NOE build-up for distance measurements

Using the homonuclear ^{1}H–^{1}H Overhauser effect (NOE) for distance measurements in macromolecules undergoing Brownian motion was the key to protein structure determination. The NOE has been in use from the early 1960s for studies of small molecules in solution [28], which are under the regime of extreme motional narrowing [23,24,29]. The major limitation of these experiments is low sensitivity for signal detection. Therefore, the NOEs were recorded in steady-state, after a preirradiation period of typically several seconds. As Kalk and Berendsen pointed out in a nice theoretical treatise [30], distance information would not be available for proteins or other macromolecules in solution when using these experiments. Intuitively, one might also have expected that the sensitivity limitation would be even more severe for proteins than for small molecules. However, a paper by Solomon [29] showed already in 1955 that the intrinsic sensitivity for ^{1}H—^{1}H-NOE observation in macromolecules is twice the sensitivity in small molecules.

In our laboratory an important advance was made when Professor Sidney L. Gordon of the Georgia Institute of Technology in Atlanta joined us for a sabbatical in 1977. Starting from a platform established by Dr. Regula Keller in my laboratory, he developed the one-dimensional transient NOE experiment and applied it with cytochrome *c* [31]. In these measurements, direct proton—proton Overhauser effects could be clearly separated from NOE intensity arising from spin diffusion, which is seen only after a lag time (Figs. 4 and 5). Applying the preirradiation pulse (Fig. 5, inset) to the well-resolved signals at both ends of the spectrum, which are shifted to the usual positions by the ring current field of the diamagnetic heme group (Figs. 1, 2, 6, 7), the one-dimensional transient NOE experiment worked beautifully for ferrocytochrome c (Fig. 5), but its application for proteins at large was limited because of extensive signal overlap in one-dimensional ^{1}H NMR spectra. However, the time course of the NOE build-up revealed by the transient NOE experiment provided a basis for the development of an alternative, the one-dimensional truncated-driven NOE (TOE) experiment [32]. TOE measurements correspond to the standard experiment used for small molecules [28], except that the preirradiation had to be highly selective for work with crowded protein ^{1}H NMR spectra; applying the CW irradiation for variable short time periods was then used to record NOE build-up curves and thus monitor the unwanted contributions from spin diffusion [32].

Collecting TOE data with short pre-irradiation times resulted in the complete assignment of the substituents of the porphyrin ring in heme c, since the NOEs enabled a step-by-step walk around the heme group (Figs. 6 and 7) [33]; these assignments were obtained without reference to the cytochrome *c* crystal structure. The earlier assignments for the iron-bound methionine side chain [4] were also confirmed and further refined [33]. Using the same approach for studies of cytochrome b_5, Regula Keller showed that the heme group in the crystal structure of this protein had to be rotated by 180° [34]. This correction of the structure was then confirmed by a new analysis of the crystallographic data. We were of course greatly encouraged by such "direct" indications that our approach

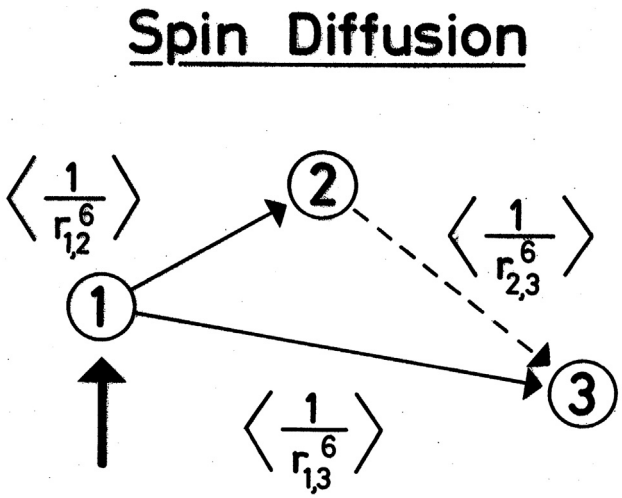

Fig. 4. Schematic visualization of spin diffusion. After build-up of coherence on atom 1 at the start of a NOE measurement (vertical arrow), transfer of magnetization to the hydrogen atom 3, in the presence of additional hydrogen atoms ("2"), goes through two competing pathways. There is the "direct" NOE across the distance $r_{1,3}$, and additional NOE intensity results from two- or multiple-step spin diffusion *via* intervening hydrogen atoms; this contribution to the overall NOE builds up after a lag period and can thus be distinguished from the direct NOE.

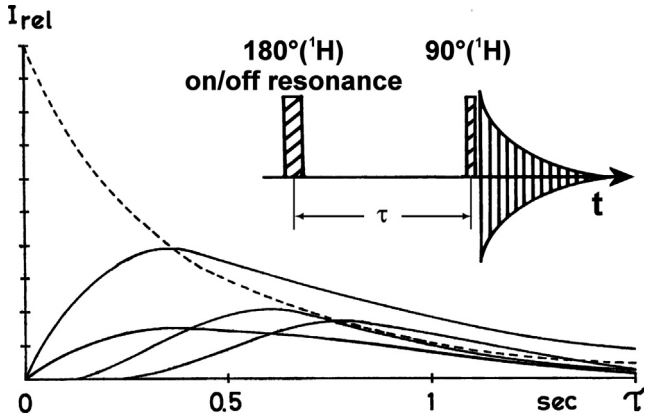

Fig. 5. 1D transient ^{1}H–^{1}H NOE measurements [31]. The inset in the upper right shows the experimental scheme used to record transient NOE spectra. A selective 180° radio-frequency pulse applied to the "pre-irradiated" ^{1}H NMR line is followed by a non-selective 90° observation pulse. The two pulses are separated by the mixing period, τ, and followed by signal acquisition. When working with the crowded spectra of proteins, we recorded the difference between two spectra obtained with the pre-irradiation 180° pulse applied on– and off-resonance, respectively. The major panel shows NOE build-up curves observed for horse ferrocytochrome c. The relative intensities, I_{rel}, of the pre-irradiated resonance line (broken curve; the pre-irradiation pulse was applied to a methyl resonance near −3.0 ppm, see Fig. 7 below) and of lines experiencing NOEs (solid curves) are plotted versus the duration of the mixing period, τ (Figure taken from [26] with permission).

for structure determination of globular proteins would indeed work out.

4.2. Solving the "phase problem" with sequence-specific NMR assignments

Having solved the problem of using ^{1}H—^{1}H NOEs for distance measurements in globular proteins under the regime of spin diffusion, the next step toward protein structure determination was to assign the measured distances to distinct pairs of hydrogen atoms. We reckoned that any number of unassigned distance constraints could not lead to three-dimensional folds of a linear chain, whereas

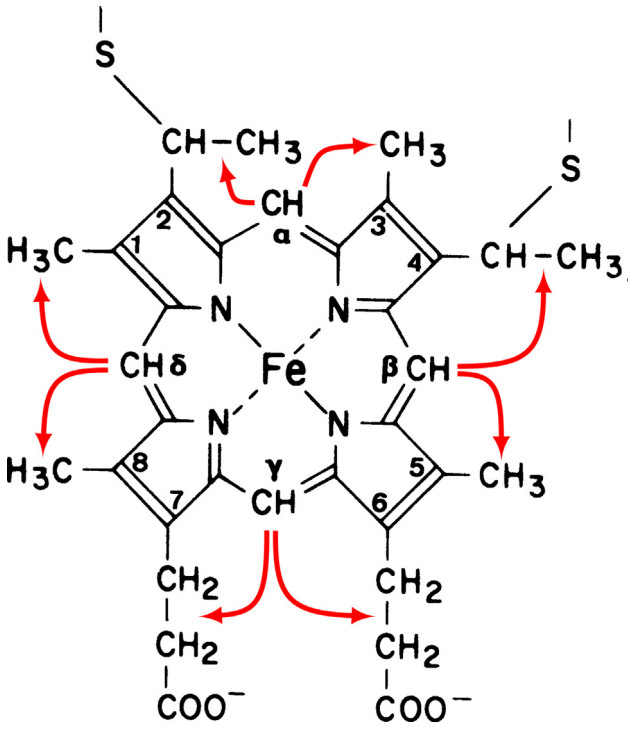

Fig. 6. Chemical structure of heme c, which is the prosthetic group in cytochromes *c*. The red arrows connect groups of hydrogen atoms in the covalent structure of heme c that are separated by a sufficiently short distance to be connected by ^{1}H–^{1}H NOEs (Figure taken from [26] with permission).

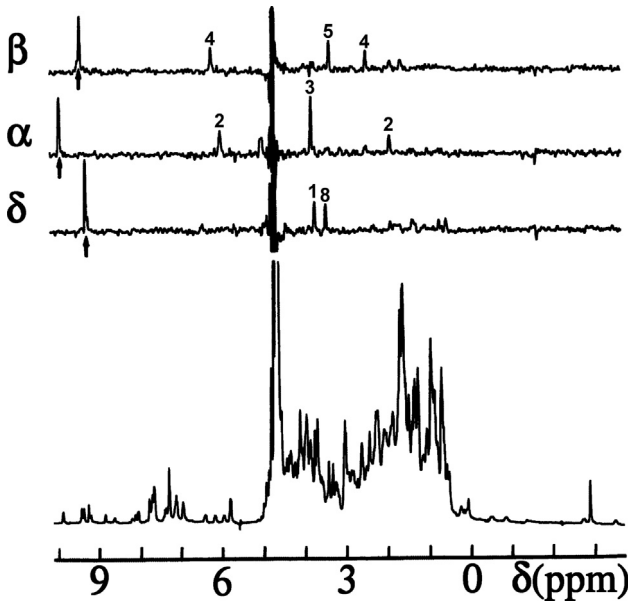

Fig. 7. Bottom trace: 1D ^{1}H NMR spectrum of ferrocytochrome c-551 from *Pseudomonas aeruginosa* [33]. Upper traces: three 1D truncated-driven ^{1}H–^{1}H NOE difference spectra [32] obtained with selective pre-irradiation (indicated by arrows) on the hydrogen atoms β, α and δ (see Fig. 6), respectively (360 MHz, 35 °C, ^2H_2O-solution). The NOE peaks are identified with numbers indicating the substituents attached to the corresponding porphyrin ring carbons (Fig. 6) (Figure taken from [26] with permission).

each assigned distance constraint defines a small or large loop that is closed by the observed short approach of two hydrogen atoms (Fig. 8). Working with small peptides at natural isotope distribution, Professor Vladimir Bystrov and coworkers at the Shemyakin

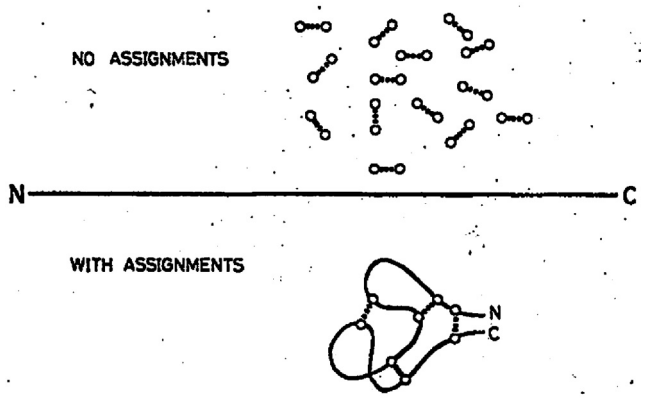

Fig. 8. Information content of ^{1}H–^{1}H NOEs in a polypeptide chain with and without sequence-specific resonance assignments. The polypeptide chain is represented by the horizontal line in the center. Pairs of polypeptide hydrogen atoms separated by a NOE-observable short distance are represented by circles connected by a solid line. With sequence-specific assignments, the NOEs enforce ring structures of variable lengths formed by the linear polypeptide chain.

Institute of Bioorganic Chemistry in Moscow had shown that the desired assignments could be obtained with the use of heteronuclear scalar spin–spin couplings [35], which is reminiscent of what is done today with triple-resonance experiments; all other successes with resonance assignments in proteins depended on reference to the corresponding crystal structures [5]. Since proteins for structural studies had to be isolated from natural sources and contained natural abundance isotope distributions for carbon and nitrogen, the Bystrov approach could not be applied with proteins, because of the low sensitivity for NMR observation of the heteronuclear spin–spin couplings at natural isotope abundance.

In our laboratory, obtaining individual NMR assignments for distinct groups of hydrogen atoms in polypeptide chains with natural isotope abundance advanced in two steps. First, a robust method was needed to connect two or several sequentially adjacent amino acid residues in polypeptide chains without prior knowledge of the three-dimensional structure. It turned out that the following strategy provided such "sequential assignments" (Fig. 9). Using 1D spin decoupling experiments to identify groups of hydrogen atoms that are related by sizeable ^{1}H—^{1}H scalar spin–spin couplings (in practice: hydrogen atoms separated by at most three covalent bonds in the chemical structure of the polypeptide fragment considered; as shown in Fig. 9, this occurs only within individual amino acid residues), we identified the proton spin systems of individual amino acid residues. Systematic investigations of the sterically allowed dipeptide fragments [8,36] then showed for all polypeptide conformations, that at least one ^{1}H—^{1}H distance between sequentially neighboring residues is sufficiently short for detection by ^{1}H—^{1}H NOEs (Fig. 9). Assignments for polypeptide segments of two to five amino acid residues in the protein BPTI were thus obtained with one-dimensional NOE experiments [37].

Considering that small proteins of 50 to 100 amino acid residues contain multiple copies of most or all of the twenty different proteinogenic amino acids, it is not a *priori* straightforward to assign sequentially connected peptide segments of two to five residues to discrete positions in the amino acid sequence. A statistical analysis of the amino acid sequences in small globular proteins then showed that repetition of identical tripeptides is very rare, and that repetition of tetrapeptide or pentapeptide segments is essentially absent. In fortunate situations, such as the *lac* repressor headpiece, a dense network of sequence-specific assignments was obtained even on the level of dipeptide segments (Fig. 10); knowing the sequence of amino acid residues between these "dipeptide anchors", the gaps could then quite readily be closed by additional "targeted" NOE experiments. Using 1D ^{1}H NMR experiments, sequence-specific assignments for the protein BPTI were obtained for the polypeptide segments with amide protons that exchanged sufficiently slowly to be observable in ^2H_2O solution (Fig. 11)

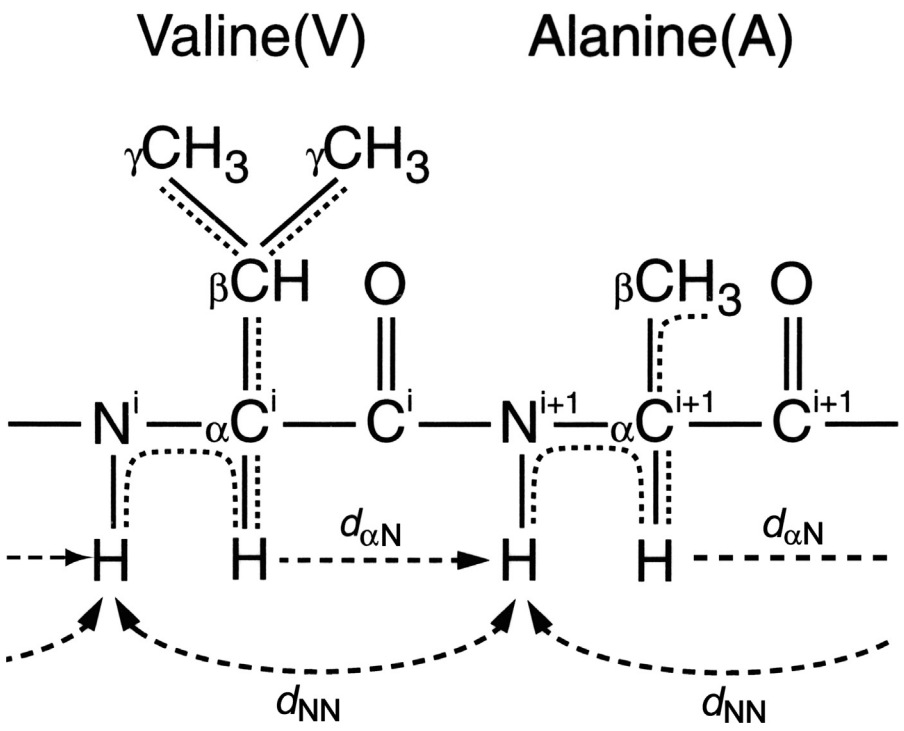

Fig. 9. Sequential ^{1}H NMR assignment of proteins. The drawing shows the chemical structure of a –valine–alanine– dipeptide segment in a polypeptide chain. The dotted lines connect groups of hydrogen atoms that are separated by at most three chemical bonds and can therefore be connected using scalar ^{1}H–^{1}H couplings. The broken arrows link pairs of hydrogen atoms in neighbouring 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" (Figure taken from [26] with permission).

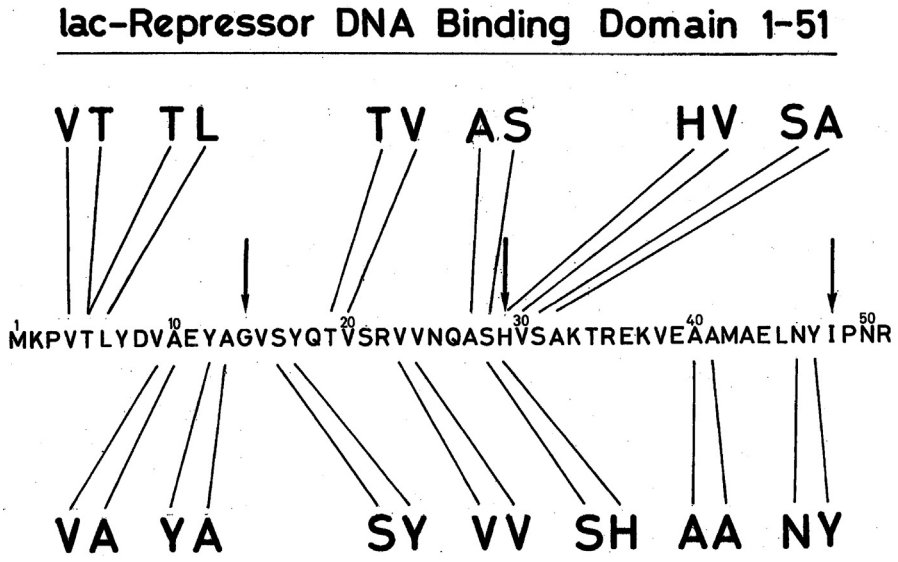

Fig. 10. Amino acid sequence of the *E. coli lac* repressor headpiece with identification of unique amino acid residues (arrows) and unique dipeptide segments (large letters). These residues and dipeptide segments served as "anchors" for obtaining sequence-specific ^{1}H NMR assignments (see text; attentive readers will notice that there are additional unique dipeptide segments in this polypeptide sequence).

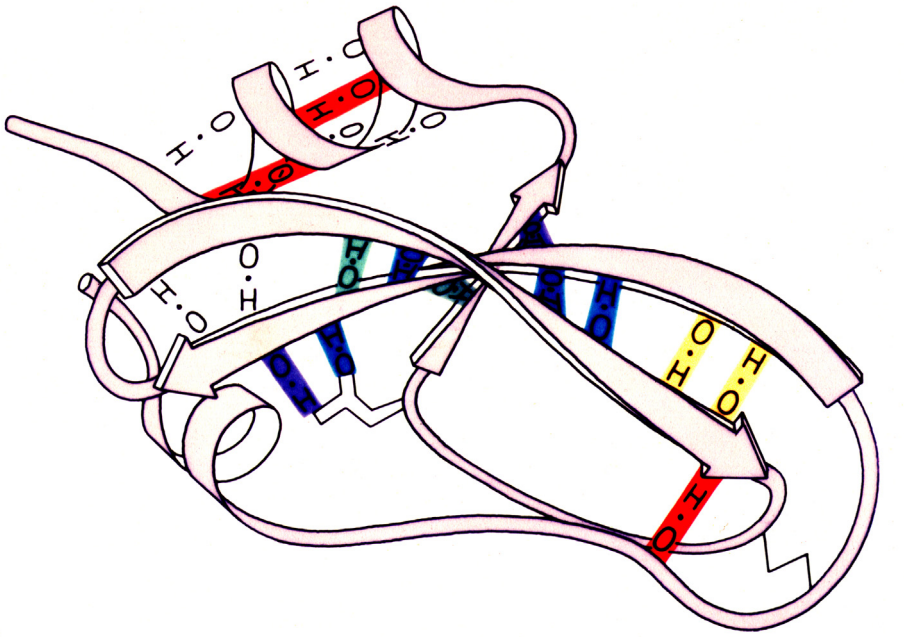

Fig. 11. Sequence-specific resonance assignments for BPTI that were obtained using 1D ^{1}H NMR experiments in $^{2}H_2O$ solution [37]. The polypeptide backbone in the crystal structure [9] is shown in a ribbon presentation, and the assigned residues are identified by the colored hydrogen bonds with their amide protons. The colour code indicates variations among the slow exchange rates of the amide protons with the solvent; blue indicates slowest exchange, and red the fastest exchange that allowed assignments in $^{2}H_2O$ solution (drawing by Jane Richardson; Figure taken from [26] with permission).

[37]. For NMR structure determination, obtaining sequence-specific assignments for the polypeptide chains in globular proteins corresponded for us to the introduction of heavy-atom clusters into proteins for solving the phase problem in the initial protein structure determinations by X-ray crystallography.

4.3. With distance geometry and NMR data to protein structures in solution

In 1978 a mathematical physicist, Dr. Werner Braun, joined my team with the goal to develop mathematical tools that would enable the calculation of three-dimensional protein structures from distance constraints that could be measured by NMR. By a fortunate coincidence, Professor Nobuhiro Go from Kyushu University in Japan joined us for a sabbatical in 1979, and he worked with Werner Braun on devising software that could provide the desired structure calculations. The starting platform was Professor Go's earlier work on the ring closure condition in cyclic peptides, which was started during a visit in the laboratory of Professor Harold Scheraga at Cornell University [38], and studies on the use of distance geometry for protein structure calculations by the Crippen and Kuntz laboratories at UC San Francisco [39,40]. The problem

to be solved was to compute three-dimensional protein structures from incomplete sets of $^1H—^1H$ distance constraints shorter than about 5.0 Å, as they can be collected by NMR. Using metric matrix distance geometry, we had to answer the following question: given upper limits and lower limits for the distances between N atoms, what are the conformations that are compatible with these distance constraints? In a polypeptide chain the upper and lower limits for the distances between covalently linked atoms are given by standard geometries for the amino acid residues. For non-bonding interactions, the lower limits are the sum of the van der Waals atomic radii, and the upper limits are obtained from the NOE measurements. In 1980 these calculations were at the limit of the most powerful computers available to us, since from the start we included all heavy atoms of the amino acid side chains; in this way we made sure that the aforementioned lower bounds would be accurately represented. This strategy was based on observations with model building, which revealed that each upper distance constraint measured by NMR generates multiple lower distance constraints, due to the close approach of atom groups imposed by the NOE data. By initially using a target function that enforced only the NOE and von der Waals distance constraints, we wanted to demonstrate that our structures were indeed the result of the NMR measurements and did not depend on refined energy minimization force fields.

The combined use of NMR spectroscopy and distance geometry was first applied for a structure determination of a nonapeptide fragment of the polypeptide hormone glucagon in perdeuterated dodecylphosphocholine (DPC) micelles [41]. This study yielded a similar result as obtained in present-day NMR structure determinations. From the residual error function value we could judge whether or not an individual distance geometry calculation converged and the resulting structure satisfied the input distance constraints. In contrast, there was no easy way to evaluate whether the input of NMR data was sufficient to result in a unique structure. We therefore decided to repeat the structure calculation with different boundary conditions; close similarity among the bundle of conformers thus obtained was then used as a criterion for accepting that the experimental data are sufficient to define a unique structure. Becasue of the limited computer facilities, the result in this very first report was presented as a bundle of only three conformers from three converged distance geometry calculations [41].

In 1982, Dr. Timothy Havel joined my group at the ETH Zürich to explore the use of the EMBED algorithm in our NMR environment. EMBED is a program for the calculation of three-dimensional protein structures from inter-atomic distances, which had resulted from Tim's Ph.D. studies at UC San Francisco [42,43]. At around the same time, Werner Braun left my laboratory for a postdoctoral assignment with Professor Go at Kyushu University in Japan. During the following years, two different distance geometry procedures were developed, DISMAN by Braun and Go in Japan [44], and DISGEO by Timothy Havel in Zürich [45]. When Werner Braun rejoined my group in 1986, we applied DISMAN and DISGEO independently for structure calculations of the protein BPTI; comparison of the results showed that protein structure determination from NMR data was robust also in the sense that identical results were obtained when using different computational routines [46].

To further explore the robustness of the method, we tested the impact of a range of input variations on protein structure determination with distance geometry calculations. The most important decision was to analyze the NOE data in terms of upper distance constraints rather than by quantative distance measurements. Depending on the signal intensity, the NOEs would correspond to upper distance limits of 3.0 Å, 4.0 Å, or 5.0 Å [47]. Another important decision for the preparation of the input for structure calculations was the treatment of groups of hydrogen atoms that could not be individually observed in the NMR spectra; examples are the methyl groups, the isopropyl groups and the symmetry-related pairs of ring hydrogen atoms of phenylalanine and tyrosine. To solve this problem, pseudo-atom positions were defined to which NOE constraints could be referenced [48]. Once software had been written that could evade local minima and result in global convergence of a protein structure calculation [45], we tested these arbitrary decisions with data sets derived from high-resolution crystal structures of small proteins. In these test runs we also systematically investigated the impact of variable completeness of the set of distance constraints used in the input. Overall, we could thus demonstrate that the metric matrix distance geometry approach is robust with regard to the interpretation of the NOEs as upper distance constraints and the use of pseudo-atoms; the sets of distance constraints could be reduced well below those obtained by NMR, provided that the remaining constraints were distributed over the entire polypeptide chain [47].

The test runs with input data derived from crystal structures confirmed the earlier finding [41] that converged structure calculations yielded a correct solution of the geometric problem to find structures that satisfied the distance constraints; this, could now be further evaluated by comparison with the crystal structure from which the constraints had been derived. However, we still did not have a generally valid criterion to decide whether with the input from NOE experiments, convergence of the distance geometry calculation yielded a correct structure. We therefore retained the earlier working hypothesis [41] to repeat the structure calculation with variable boundary conditions. At this early stage only about one in five structure calculations resulted in global convergence, as judged by the residual value of the target function; if all converged structure calculations yielded similar results, we assumed that we had determined a unique structure.

4.4. Two-dimensional NMR

The development of 2D NMR with biological macromolecules, which resulted from a collaboration with Professor Richard R. Ernst, made protein structure determination by NMR a viable method of structural biology. This work was pursued in a most invigorating environment, since magnetic resonance spectroscopy was a very important part of physics and chemistry research at the ETH Zürich and the University of Zürich; in the 1970s, NMR was represented by more than ten full professors. There was also the rapidly evolving Bruker-Spectrospin company, which has its origins in a spin-off of the ETH Zürich based on pioneering work by Professors Hans H. Günthard and Hans Primas at the Laboratory of Physical Chemistry.

Richard Ernst and I first met in person on October 1, 1969, when I returned from my employment at Bell Telephone Laboratories to Switzerland and reported for work at the ETH Zürich. I was formally a member of the Institute of Molecular Biology and Biophysics. Since there was a delay with the construction of a home for this new institute, Professor Günthard had arranged for me to have a workplace and access to NMR instrumentation in the Laboratory of Physical Chemistry. Richard Ernst was in charge of a superconducting NMR spectrometer operating at 220 MHz, which had been installed shortly before my arrival as a service facility for the chemistry laboratories of the ETH Zürich and the University of Zürich. I was one of the users of the 220 MHz spectrometer, and initially the contacts with Richard Ernst were mainly focused on optimizing the operation of this CW instrument. In the Fall of 1970 I moved from the Laboratory of Physical Chemistry in the city center to a new building of the Hönggerberg campus of the ETH Zürich, where my laboratory was equipped with 60 MHz and 100 MHz NMR spectrometers and an X-band EPR spectrometer. During the following years, my group continued to make extensive

use of the 220 MHz spectrometer, and we met almost weekly with Richard Ernst and members of his team. Attendance at the Ernst lecture course on "Advances in Magnetic Resonance" was a must for all graduate students and postdoctoral fellows in my group, and there were occasional joint group seminars. In collaboration with Professor Joachim Seelig of the University of Basel, Richard and I then organized the 1974 ICMRBS in Kandersteg; we also took the initiative to organize a first "Swiss NMR Symposium", which has become a bi-annual event. At the 1974 ICMRBS, Richard Ernst presented the first two-dimensional NMR experiments, and his group followed this up with key developments in 2D NMR with small molecules [49].

A joint research project with Richard Ernst was funded in 1976 under the auspices of the Swiss Commission for Technology and Innovation ("Kommission für Technologie und Innovation **KTI**"). In hindsight it is interesting that the Swiss National Science Foundation, which supports "basic research", transferred our project to the KTI, which supports "applied research" in collaboration with industry. Our industry partner was the nearby Bruker-Spectrospin company. By then, our research groups had individually grown from modest beginnings to sizes of about 20 scientists each. Quite Independent of the other activities in our research groups, this new venture was dedicated to transforming multi-dimensional NMR from an intellectually exciting breakthrough [49] to a technique that could be used in structural biology as well as in chemistry. Small teams of Richard Ernst, Kuniaki Nagayama, Peter Bachmann and myself during the first three years, and Richard Ernst, Anil-Kumar, Gerhard Wider, who was at the time one of my graduate students, and myself for an additional year, developed the ability of recording homonuclear 2D ^{1}H NMR spectra with large data matrices (Fig. 12). Four key experiments, 2D spin-echo-correlated spectroscopy (SECSY) [50], 2D correlated spectroscopy (COSY) [51], 2D nuclear Overhauser spectroscopy (NOESY) [52] and 2D fold-over-corrected correlated spectroscopy (FOCSY) [51,53], enabled the collection of the data needed for the initial protein structure determinations. The software developed in this project was written for the dedicated computers that were part of Bruker Instruments; they were distributed by Bruker-Spectrospin to their customers, initially without any adaptations. A key experiment for protein structure determination was the measurement of NOE build-up rates with [^{1}H,^{1}H]-NOESY [55]. This experiment (Fig. 13) combined the experience from 2D exchange spectroscopy (EXSY) in the Laboratories of Professor Jean Jeener at the Université Libre de Bruxelles and of Richard Ernst [54], our experience with one-dimensional NOE experiments [31,32], and the ability to record 2D [^{1}H,^{1}H]-NOESY experiments with large data matrices [50–53]. With this approach, we could now efficiently measure ^{1}H—^{1}H distances in macromolecules in solution.

We faced some practical difficulties during the four years of this project, which would not need to be considered today. First, the

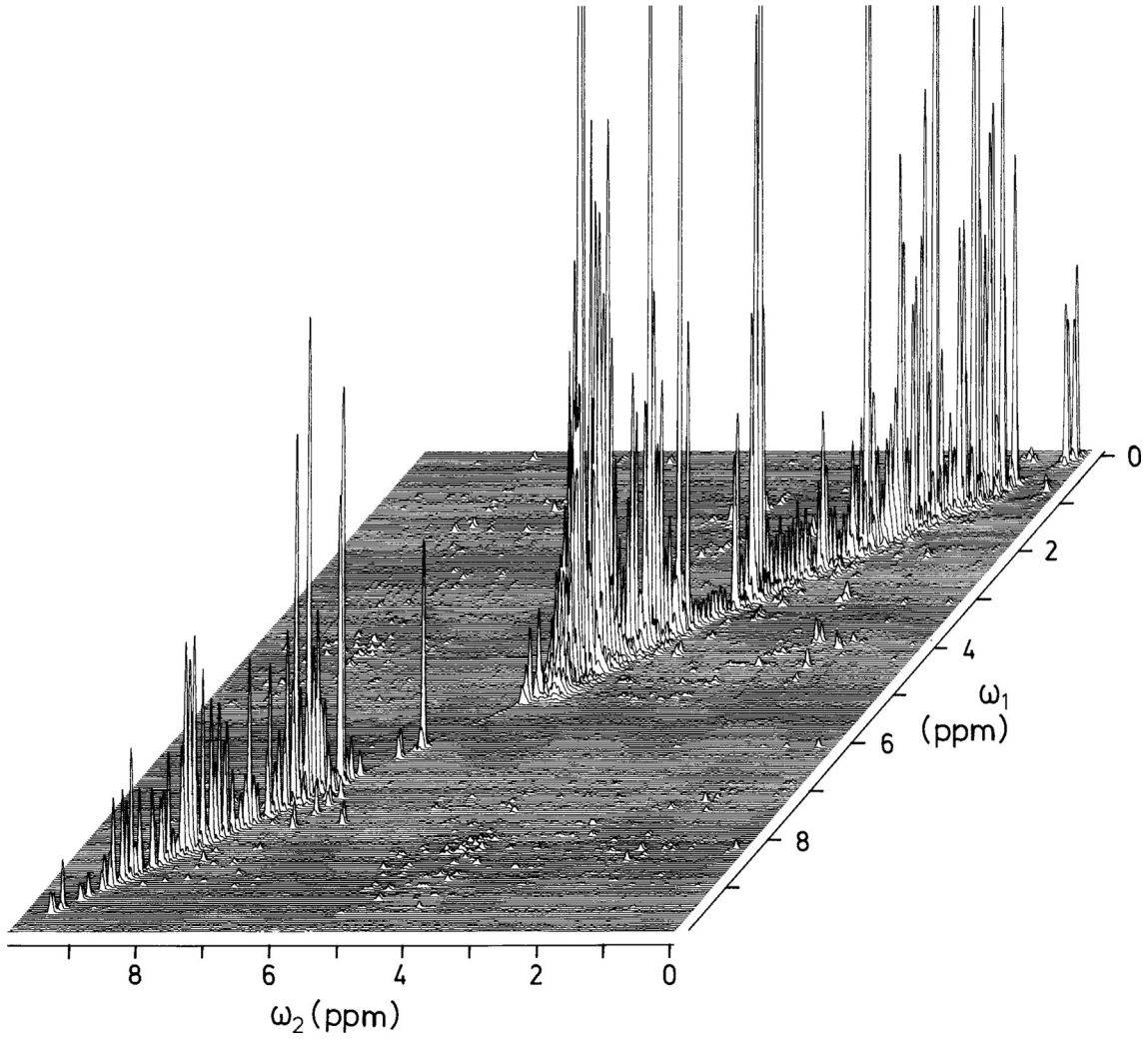

Fig. 12. 2D [^{1}H,^{1}H]-NOE spectroscopy ([^{1}H,^{1}H]-NOESY). A stacked plot representation of a spectrum of the protein BUSI is shown (500 MHz, 45 °C, H_2O-solution) (Figure taken from [26] with permission).

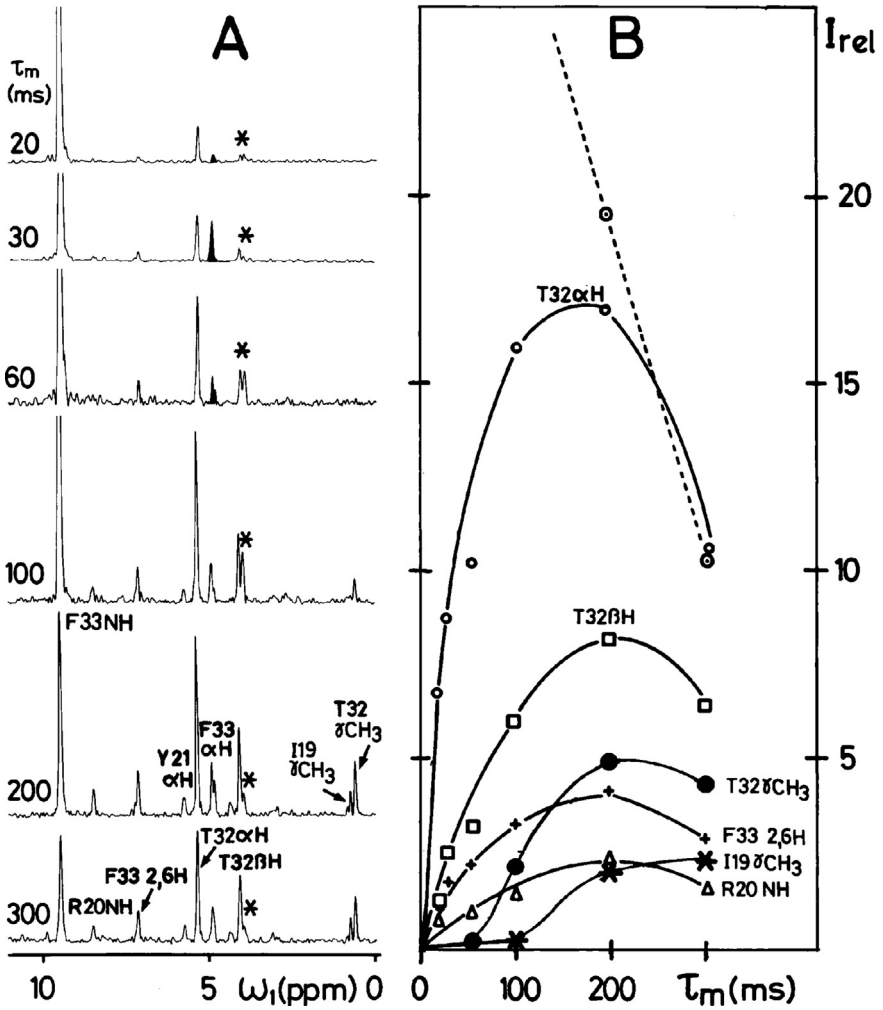

Fig. 13. Measurement of NOE build-up curves using 2D [^{1}H,^{1}H]-NOESY experiments. (A) Six NOESY spectra of BPTI recorded with different mixing times, as indicated on the left. The same 1D cross-section along the ω_1 frequency axis through the diagonal peak of the Phe 33 amide proton is plotted for each spectrum. (B) NOE build-up curves obtained from the analysis of the data in (A). Relative peak intensities are plotted versus the mixing time, τ_m; the broken line represents the decay of the magnetization on the diagonal peak of the Phe 33 amide proton, and the solid lines show the evolution with τ_m of the NOESY cross peaks of nearby hydrogens in the BPTI structure (Figure taken from [26] with permission).

data matrices generated by high-resolution 2D ^{1}H NMR experiments at high fields were very large by the standards of the informatics equipment available in the late 1970 s. To overcome this difficulty, folded versions of all the 2D NMR experiments were devised. Once improved computer equipment became available, interest was lost in the folded experiments, for example, SECSY and FOCSY. Second, once the software was ready to record initial spectra, we discovered that the homogeneity of the radiofrequency pulses was insufficient for homonuclear 2D ^{1}H NMR, and new hardware (initially "cavity probeheads") was then developed at Bruker-Spectrospin; this illustrates the continuous important contributions of the industry partner in the 2D NMR project. Third, the lines in the two-dimensional data sets were very broad, and useful frequency-domain spectra were obtained only after extensive data handling, primarily with sinusoidal window functions [56] and symmetrization [57].

4.5. Protein structures from homonuclear 2D ^{1}H NMR and distance geometry

As described in the preceding sections, the strategy for *de novo* protein structure determination by NMR in solution was generated on the basis of one-dimensional ^{1}H NMR experiments, but it was the introduction of 2D ^{1}H NMR that made protein structure determination in solution a viable method. The impact of 2D NMR is readily seen from comparison of the Figs. 11 and 14. The sequence-specific resonance assignments for BPTI obtained with 1D ^{1}H NMR (Fig. 11) were limited to the amino acid residues with the most slowly exchanging amide protons in this outstandingly stable protein. For all other residues, the amide protons had been exchanged with deuterium. The residual spectrum in the amide proton region was sufficiently well resolved for the application of selective pre-irradiation in TOE experiments [32]. In contrast, 2D^{1}H NMR experiments were recorded in H_20 solution, and almost complete (see below) sequence-specific resonance assignments were obtained for BPTI. These then enabled us to collect a sufficient number of NOE distance constraints for the calculation of the three-dimensional structure [46].

By the end of 1980, the four homonuclear 2D ^{1}H NMR experiments COSY, SECSY, FOCSY and NOESY were available for use by all scientists in my group, and we obtained sequence-specific resonance assignments for numerous small proteins (for example, [58–64]). In 1982 we published four consecutive papers in the *Journal of Molecular Biology*, with the introductory title "Sequential Resonance Assignments as a Basis for Determination of Spatial Protein Structures by High Resolution Proton Nuclear Magnetic Reso-

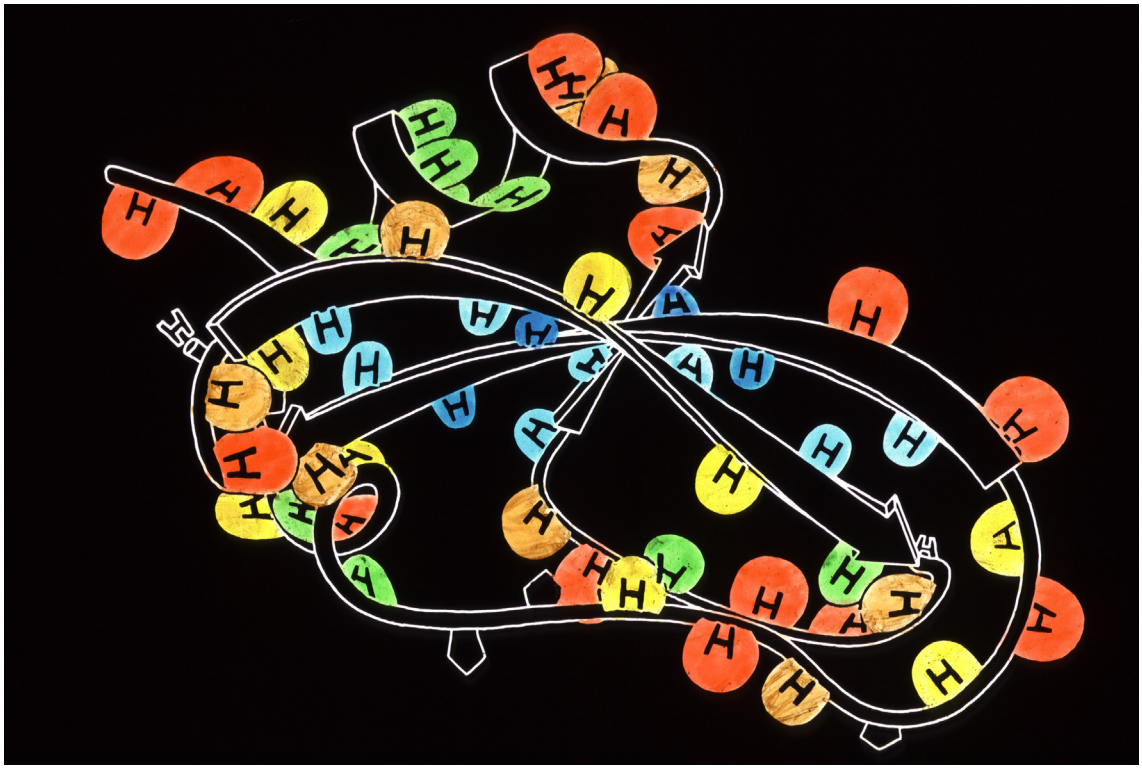

Fig. 14. Sequence-specific resonance assignments for BPTI obtained using 2D ^{1}H NMR experiments. Assigned residues are identified by colored shapes around their amide protons. The colour code indicates variable amide proton exchange rates, with blue indicating slowest exchange, and yellow and red identifying amide protons that could be observed only in H_2O solution (drawing by Jane Richardson; Figure taken from [26] with permission).

nance" and a total volume of 77 pages. The four publications describe the foundations for obtaining sequence-specific resonance assignments and describe complete sets of resonance assignments for BPTI and for glucagon bound to DPC micelles [65–68]. Having thus solved the NMR assignment problem, a structure of micelle-bound glucagon was completed [69] (see below), but it took two more years for the development of improved algorithms and their implementation for distance geometry calculations [44,45,47] that could yield the first complete structure determination of a globular protein, bull seminal protease inhibitor (BUSI) [1].

During the last phase of the methods development for protein structure determination by NMR in solution, from 1981 to 1985, we worked with glucagon bound to DPC micelles, BPTI, BUSI and metallothionein (MT). However, these four years were full of excitement, and there were many additional projects ongoing. Intense work, mostly in collaboration with Richard Ernst, was focused on new NMR methodology. Numerous scientists of the Ernst and Wüthrich research teams now contributed to these projects, in addition to the single postdoctoral associate that we could hire on the joint grant. The new NMR experiments subsequently had important roles in continued improvement of multi-dimensional NMR for chemistry and structural biology, but with two important exceptions they did not bear on the initial structure determinations. The exceptions are the developments of heteronuclear ^{113}Cd—^{1}H correlation spectroscopy [70] and of phase-sensitive COSY [71], which were crucial for the structure determination of metallothionein (see below).

The four "work horses" for the protein structure determination project had different important roles. The polypeptide hormone glucagon consists of a 29-residue polypeptide chain, which does not adopt a globular fold. By attaching this polypeptide to fully deuterated DPC micelles, the molecule was under a similar regime of Brownian motions as small globular proteins, while it retained the comparatively simple spectrum corresponding to its small size. For this reason, an initial partial structure determination could be pursued with 1D ^{1}H NMR experiments [41]. With the use of 2D ^{1}H-NMR, a complete structure was obtained in 1983 [69]. Thereby, the structure calculation could only be performed for multiple overlapping nonapeptide segments, which was the size limit for our initial distance geometry program [41]; while we considered that this "segmental" approach was viable for non-globular polypeptides, it was not applicable for globular proteins.

BPTI was used for most of the development work on 1D and 2D ^{1}H NMR spectroscopy as well as on structure calculation from NMR data. Since a high-resolution crystal structure was available [9], it was not an attractive candidate for documenting that *de novo* structure determination by NMR worked out. Furthermore, a tetrapeptide segment of the polypeptide chain was not represented in the NMR spectra. We eventually published the BPTI structure determination as a techniques paper [46]; the NMR structure was closely similar to the previously determined crystal structure [9]. A decade later we discovered that the missing NMR lines were broadened beyond detection by low-frequency intramolecular rate processes [72].

BUSI, the first protein for which a NMR structure was published [1], was provided to us by the laboratory of Professor Dana Cechova at the Czechoslovak Academy of Sciences in Prague, when Dr. Petr Strop from her group joined us as a postdoctoral fellow. This protein was a good candidate; while no crystal structure was available, we could compare our results with crystal structures of homologous proteins [1]. BUSI yielded high-quality NMR spectra that represented the entire polypeptide chain, and all steps of the structure determination went smoothly [1,63,73–75]. At the time we felt that it was important to document the application of the structure determination method developed using glucagon and BPTI with a new protein.

The metal-containing mammalian MT proteins have highly unusual amino acid sequences; the 62-residue polypeptide chain

of the rabbit metallothionein contains only 12 different amino acids, including 20 cysteines, 9 alanines, 8 serines and seven lysines, which made the sequence-specific assignment a real challenge [76,77]. Prior to our work with this protein, it had been known that mammalian MTs contain two metal clusters of, respectively, four and three Zn (II)-ions. In collaboration with Professor Jeremias Kägi of the University of Zürich, the zinc ions were replaced with ^{113}Cd. With the use of the aforementioned heteronuclear correlation experiment [70], a total of 28 coordinative bonds between the seven metal ions and the sulfur atoms of the 20 cysteines were identified, showing that the structure includes eight "bridging cysteines" that are bound to two metal ions. The three-dimensional fold of the polypeptide chain, which forms two flexibly linked globular domains, was then determined with the same approach that was used for the other proteins [78,79].

5. Results from protein structure determination by NMR solution

NMR determination of complete three-dimensional protein structures was initially a slow process, and obtaining intermediate results was an important lifeline. It turned out that ^{1}H NMR data of globular proteins can in straightforward ways be related to the amino acid sequence as well as to regular secondary polypeptide structures, such as helices and sheets, since these are both intimately linked with obtaining sequence-specific resonance assignments by ^{1}H NMR.

Regarding the primary structure, it was common during the early applications of the sequential assignment method that we detected errors in amino acid sequences that had been determined by chemical analysis of the polypeptide chains. The way to checking on the chemically determined amino acid sequence is readily apparent from Figs. 9 and 10. Once a network of sequence-specific assignments for short polypeptide segments had been obtained (Fig. 10), the intervening gaps were closed by NOE-guided walks along the chemically determined sequence (Fig. 9). Since the individual proteinogenic amino acids have different ^{1}H spin systems, deviations from the previously determined amino acid sequence would then become apparent. Typically, we would find between zero and five errors in proteins with sixty to eighty residues. During the first three decades of structural biology, X-ray crystallography has, in a similar way, contributed to amino acid sequence determination.

Sequential resonance assignments by ^{1}H NMR and the identification of regular secondary structures rely on the same ^{1}H NMR data, so that the sequence-specific ^{1}H NMR assignment for the polypeptide chain in a globular protein results also in the identification of polypeptide segments that form regular secondary structures. Analysis of the ^{1}H—^{1}H distances within pentapeptide segments of polypeptide chains revealed that in helices and sheets, respectively, different pairs of hydrogen atoms are related by short distances [36]. Uniform distance distributions along polypeptide segments of five or more amino acid residues therefore identify the presence of regular helical or sheet secondary structures (Fig. 15). Supplementary information is provided by the $^3J_{HN\alpha}$ spin–spin couplings. In Fig. 15 the locations of α-helices are identified from continuous runs of small $^3J_{HN\alpha}$ values and intense d_{NN} NOEs (Fig. 9), and independently from the presence of medium-range NOEs, which are seen only in helical structures.

In the early 1980′s the presentation of the three-dimensional protein structures was still in development (for example, [80]), and the computer drawings shown here were prepared many years later (the hand-drawings by Jane Richardson in Figs. 11 and 14 date from 1979 and 1981, respectively). As was discussed above, the results of NMR structure determinations were from the beginning presented as bundles of conformers superimposed for minimal root mean square distance (RMSD) of the backbone heavy atoms. The Fig. 16 shows the polypeptide backbone of the *Antennapedia* homeodomain, which has a well-defined central region and two disordered chain ends. While the structure determination shows that the chain ends are not well defined in the protein architecture, additional NMR experiments were needed to demonstrate that the chain ends are flexibly disordered. Similar observations can be made with BPTI in Fig. 17, where the polypeptide backbone and the interior amino acid side chains are well defined by the NMR data, whereas the solvent-accessible amino acid side chains on the protein surface and the polypeptide chain ends are poorly defined; again, additional studies showed that the disorder is highly dynamic. In presentations of the results from a NMR structure determination, one typically includes a representative single conformer from the bundle; as an illustration, Fig. 18 shows BUSI, which was the first complete NMR structure of a globular protein [1}.

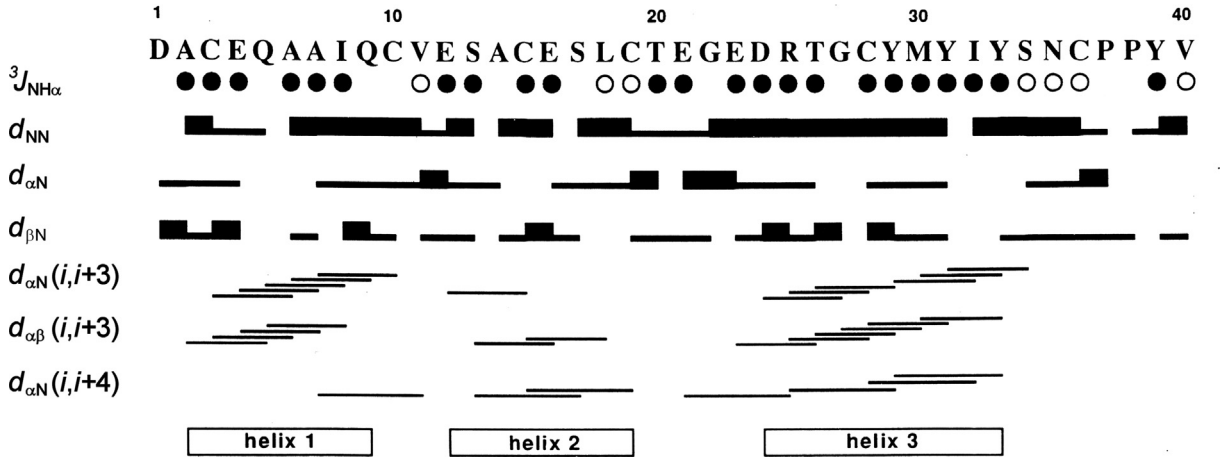

Fig. 15. ^{1}H NMR data for sequence-specific resonance assignments and identification of regular secondary structures in the pheromone protein Er-1 from *Euplotes raikovii*. In the first row below the amino acid sequence, filled and empty circles identify small and big values of the spin–spin coupling constants $J_{HN\alpha}$. d_{NN}, $d_{\alpha N}$ and $d_{\beta N}$ are distances manifested by sequential NOEs (Fig. 9), where the thickness of the lines represents the intensity of the NOEs. 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 by 3 or 4 sequence positions; in the drawing the observation of medium range NOEs is represented by horizontal lines. The locations of three α-helices identified by the NMR data are indicated at the bottom (Figure taken from [26] with permission).

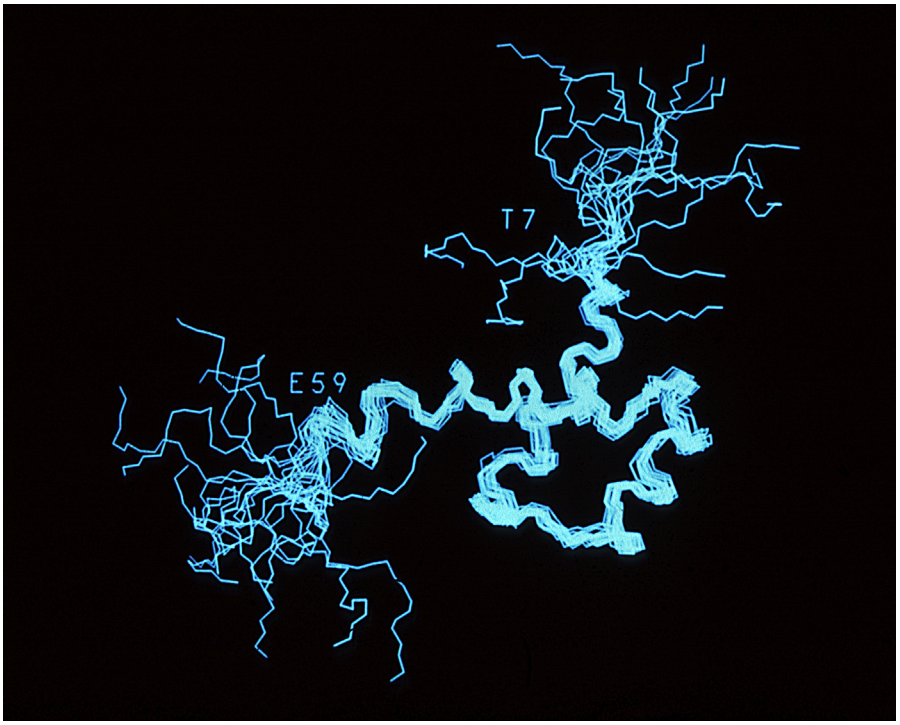

Fig. 16. NMR structure of the *Antennapedia* homeodomain. A bundle of 20 superimposed conformers represents the polypeptide backbone. For the polypeptide segment 7–59 the tight fit of the bundle indicates that the structure is defined with high precision, whereas the two chain ends are disordered (Figure taken from [26] with permission).

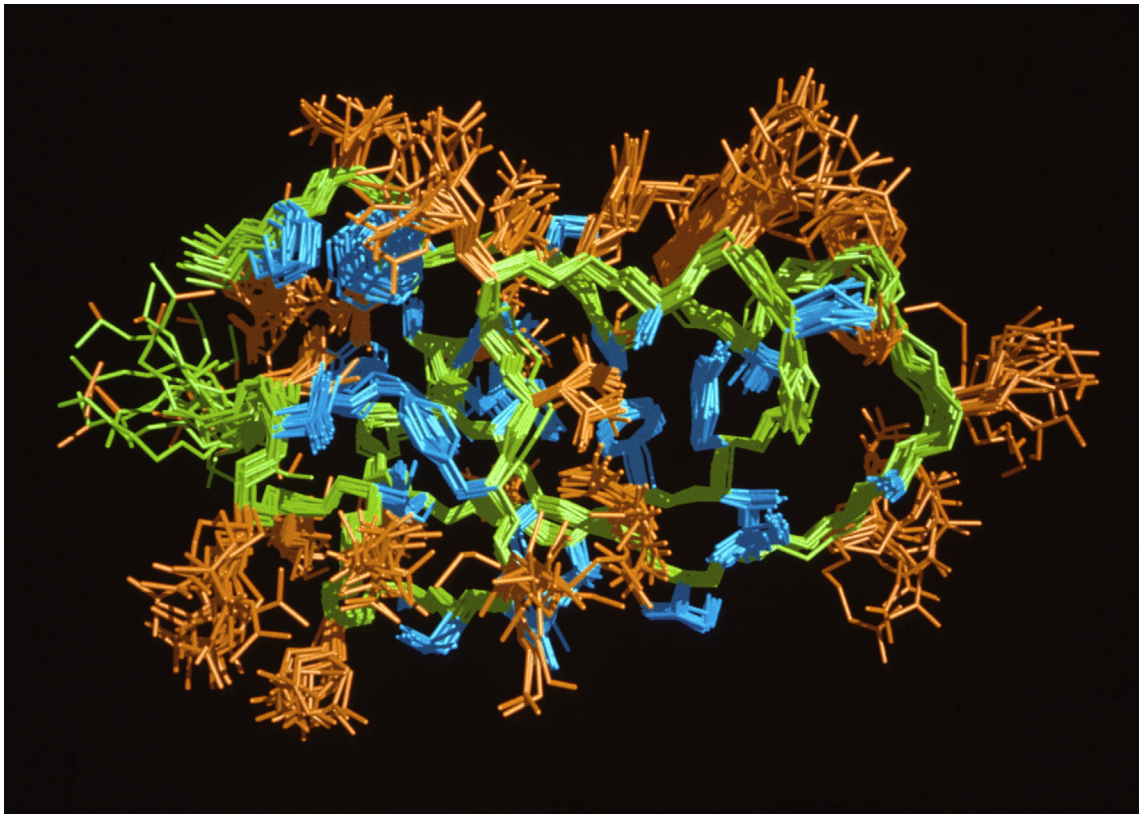

Fig. 17. All-heavy atom visualization of the NMR structure of BPTI represented by a bundle of 20 conformers superimposed for best fit of the polypeptide backbone. The polypeptide backbone is green, core side-chains are blue, and solvent-accessible surface side-chains are red (Figure taken from [26] with permission).

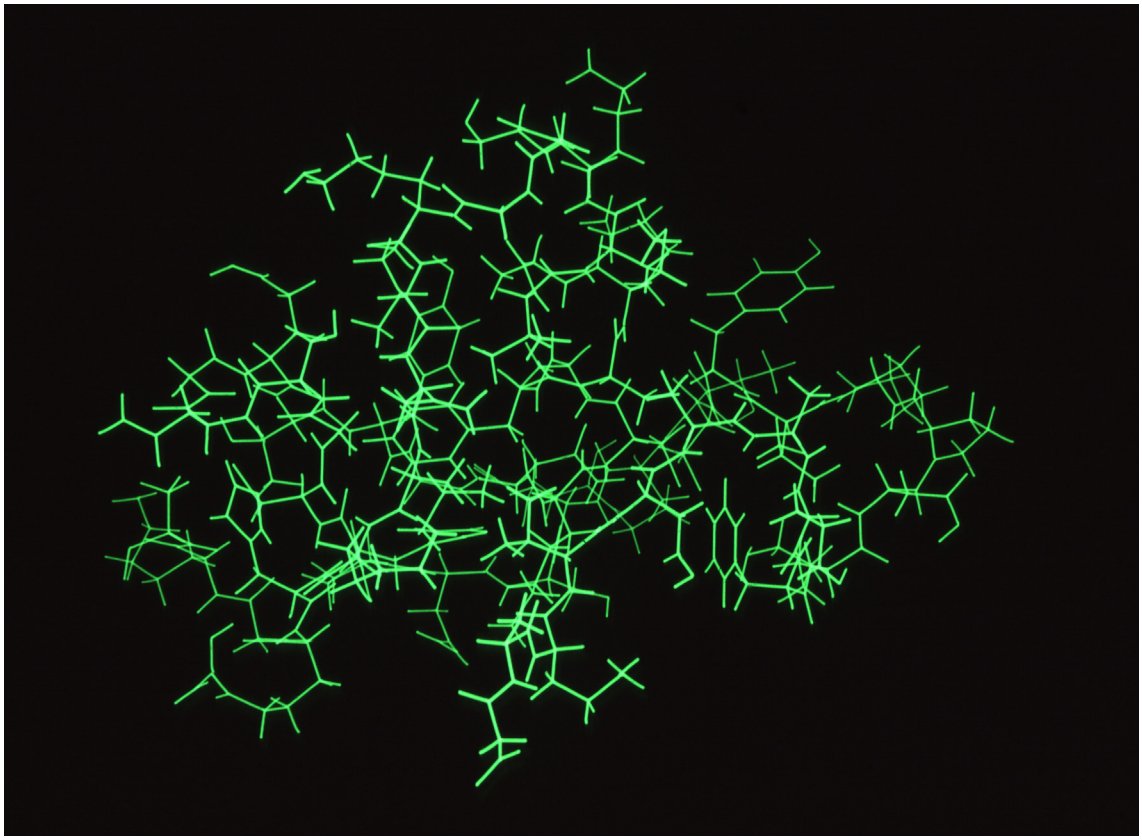

Fig. 18. The NMR structure of BUSI published in 1985 [1] is represented by the "best" conformer out of a bundle of results from converged structure calculations as in Fig. 17 (Figure taken from [26] with permission).

6. Introducing NMR structures to the structural biology community

In the summer of 1985 I faced heavy criticism after presentations of the first two NMR structures of globular proteins in congresses and universities in Europe and the USA. The structural biology establishment of the mid-1980s was convinced that it was *a priori* not possible to get an atomic-resolution image of a protein in solution at ambient temperature, where it is under the regime of Brownian motion, i.e., undergoing stochastic translational and rotational movements at frequencies of about 10^8 sec^{-1}; most of the colleagues working with protein crystals had not appreciated that NMR structure determination in solution is based on measurements of scalar parameters that are invariant under translational and rotational motions. We had proudly taken the close fit of our NMR structure of BUSI with the crystal structure of a homologous protein in the protein data bank (PDB) as yet another proof that the NMR method worked [1]. For others, it raised doubts on whether we really presented a structure based solely on NMR data.

With metallothionein (MT), an additional problem surfaced: at Yale University, a NMR structure of rat MT was presented to me that was widely different from our rabbit MT structure, and at the University of Pittsburgh I was introduced to a crystal structure of rat MT that was completely different from our NMR structure [81]. Rabbit MT and rat MT have nearly identical amino acid sequences, which include the 20 cysteines in identical sequence positions, and they both bind seven divalent metal ions, Zn(II) and/or Cd(II). Among 28 metal–cysteine coordinative bonds, which include 8 "bridging cysteines", 21 bonds were different between this crystal structure and our NMR structure (they were yet different in the NMR structure at Yale). For many (not for all; for example, one of the early champions of protein crystallography, Professor Frederic M. Richards at Yale University, outspokenly supported our structure) this demonstrated that the NMR approach was not able to determine a three-dimensional protein structure *de novo*. After learning in Pittsburgh about the crystal structure of rat MT, I called Gerhard Wagner in Zürich to re-check the NMR assignments on which our structure determination was based. As mentioned above, the unusual amino acid composition made sequence-specific NMR assignments for this small protein a real challenge. After several hour-long telephone calls out of my hotel room, with both of us staring at the original data (of which I had carried a copy with me), we found that there were no errors. Gerhard felt deeply insulted by my having even considered the possibility of him having made mistakes; it was a hard test for our long-standing friendship. Second, an editor of *Science* decided that the crystal structure of rat MT must be correct, because "crystallography was a proven method for protein structure determination" [81]. We were left with difficult interactions with editors and reviewers, which resulted in long delays of the publication describing our structure determination of rabbit MT [79]. Before our structure of rabbit MT was accepted for publication, we had to demonstrate that the NMR structure was not an artefact of the reconstitution needed to replace the Zn(II) ions with the NMR-active ^{113}Cd [82]. We also had to determine the NMR structure of rat MT and show that it is different from its crystal structure [81] and nearly identical to the rabbit NMR structure [83,84]. Finally, the crystal structure was re-determined in the early 1990s. In a joint publication with two authors of the *Science* paper that presented the original, misleading crystal structure [81], we reported that the new crystal structure was identical to the NMR structure that we had determined in 1985 [85]; this paper was eventually published in PNAS, because the editor of *Science*

refused to publish this story, which corrects an earlier erroneous *Science* paper[81] that was never retracted.

As an immediate reaction to a lecture on the BUSI NMR structure, Professor Robert Huber at the Max Planck Institute for Biochemistry in Martinsried suggested in the summer of 1984 that we determine a novel protein structure in parallel by crystallography and by NMR, with the aim of comparing the two structures and thus get an "unambiguous proof" of whether the NMR method could provide *de novo* atomic-resolution protein structures. The company Hoechst AG provided each one of us with 100 g (*sic*!) of the α-amylase inhibitor Tendamistat. Dr. Allen Kline, who had just joined my team, agreed to accept this challenge. After selecting the best out of several hundred solution conditions for the NMR experiments, we published a complete description of the secondary structure on January 2, 1985. This included subtle details, such as the identification of β-bulges [10] in the otherwise regular β-sheets [86]. This encouraging result provided the basis for the complete NMR structure determination, which was available before Tendamistat could be crystallized [87,88]. Once the crystal structure was also available, it turned out that the two structures were identical in the global fold as well as in subtle local structural traits [89], and the X-ray structure of Tendamistat was eventually redetermined with a molecular replacement approach that used the NMR structure to solve the phase problem [90]. At this point, protein structure determination by NMR in solution got quite generally accepted, and we also learned through the grapevine that a new crystal structure determination of rat MT was started (see above and [85]). The editor of the *Journal of Molecular Biology*, Dr. Sydney Brenner, added the following comment at the end of the 50-page paper on the Tendamistat NMR structure determination [88]: *"We have taken the step of publishing this paper with full supporting data since it is the first high resolution structure worked out in detail by 2-D NMR. We therefore think that in this one instance everything should be published in full, but it does not set a precedent, since it is hoped that in the future, such supporting data can be deposited in a data bank, as is the practice in X-ray protein crystallography."*

7. Afterthoughts

While the above quotation of Dr. Sydney Brenner is a fitting conclusion for a historical account of the introduction of protein structure determination by NMR in solution to the 1980s structural biology community, the reviewers kindly suggested that I should add a few sentences to place the history piece in a wider frame of reference. Here is my response.

On June 17, 2021, at 16:00 h CEST, the Protein Data Bank (PDB) listed 13,415 NMR structures that have been deposited since 1985. These entries document the wide range of front-line biological and biomedical research that has been advanced by NMR structure determination of proteins, nucleic acids and higher-order supramolecular assemblies. From the mid-1980s, biological and biomedical applications became the principal line of research also in my laboratory; these applications then continuously motivated methods developments to respond to new, application-based demands. In collaborations with friends and colleagues who contributed the expert biochemistry of their projects, we addressed widely different areas; to name just a few, we studied differentiation in higher organisms [91,92], immune suppression [93,94], prion proteins and prion diseases (transmissible spongiform encephalopathies such as Creutzfeldt-Jacob disease in humans and "mad cow disease" in cattle) [95–97], signaling by pheromones [98–101], and information transfer across cell membranes by G protein-coupled receptors (GPCRs) [102,103]. Some of our early structure determinations were successful competitions with X-ray crystallography; examples are the *Antennapedia* homeodomain [91] and the prion protein [95], wich both had for many years resisted attempts at crystal structure determination. With time, complementing of macromolecular structures determined by other methods has become the main focus of our work [94,104,105], which includes integrative projects under the auspices of structural genomics programs [106–109].

In addition to telling us about the impact of NMR structure determination of biological macromolecules on biochemical, biological and biomedical research, the 13,415 listings of NMR structures in the PDB are a testimony of the wealth of methods developments that have been added to each step of the protocol for protein structure determination by NMR. This includes new biochemistry for the sample preparation, new concepts in NMR spectroscopy, and overwhelming advances in computer hardware and software. It is a great pleasure for me to follow the continuing advances in NMR methods and their impact on the front-lines of natural sciences and human medicine.

Declaration of Competing Interest

The author declare that there is no conflict of interest.

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