Structure Determination of Polypeptides and Proteins by Two- Dimensional NMR Spectroscopy

ARTICLE *in* PHYSICA B CONDENSED MATTER · JUNE 1990

Impact Factor: 1.32 · DOI: 10.1016/0921-4526(90)90074-5

3 AUTHORS:

Hans Robert Kalbitzer
Universität Regensburg
300 PUBLICATIONS **5,359** CITATIONS
SEE PROFILE

Klaus-Peter Neidig
Bruker Corporation
17 PUBLICATIONS **430** CITATIONS
SEE PROFILE

Wolfgang Hengstenberg Hengstenberg
Ruhr-Universität Bochum
134 PUBLICATIONS **3,151** CITATIONS
SEE PROFILE

Physica B 164 (1990) 180–192
North-Holland

STRUCTURE DETERMINATION OF POLYPEPTIDES AND PROTEINS BY TWO-DIMENSIONAL NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Hans Robert KALBITZER, Klaus-Peter NEIDIG and Wolfgang HENGSTENBERG[a]
Max-Planck-Institut für medizinische Forschung, Abt. Biophysik, Jahnstr. 29, D-6900 Heidelberg, FRG
[a]*Ruhruniversität Bochum, Abt. Biologie, D-4630 Bochum, FRG*

Determination of the three-dimensional structure of small, soluble proteins by two-dimensional nuclear magnetic resonance (NMR) spectroscopy represents an alternative method of X-ray crystallography and is an important application of high magnetic fields in science. The basic methodology is introduced and shortly reviewed, limitations and possible future developments are discussed.

1. Introduction

Nuclear magnetic resonance (NMR) has developed to one of the most important applications of high magnetic fields in science. For more than twenty years NMR spectroscopy is an established analytical tool in chemistry, in recent years it has grown more and more important in biosciences and is now one of the most often cited physical methods in biochemistry (for monographs see, e.g. refs. [1–4]. For many biochemical applications the highest magnetic field strength available is required, since sensitivity and resolution increase with the magnetic field. The highest magnetic field commercially available and satisfying the requirements of high resolution NMR (field drift of less than 0.03 ppm/h without external stabilization, homogeneity over a typical sample diameter of 0.5 to 1 cm better than 0.3 ppm/cm without room temperature shim system and better than 0.0003 ppm/cm with room temperature shim system and sample rotation) is 14.1 T corresponding to a proton resonance frequency of 600 MHz. Today, more than thirty 600 MHz NMR spectrometers and more than two hundred 500 MHz NMR spectrometers are installed worldwide.

There are three relatively new developments of NMR in biosciences, magnetic resonance tomography including NMR microscopy, in vivo NMR spectroscopy and, last but not least, determination of the three-dimensional structure of biological macromolecules by two-dimensional NMR spectroscopy.

Knowledge of the spatial structure of a macromolecule is a prerequisite for a deeper understanding of its function, since the three-dimensional arrangement of the atoms in the macromolecule determines its physical–chemical as well as its biological properties.

From the many types of macromolecules occurring in biological systems there are three important groups of molecules composed of only a limited number of different building blocks, the polypeptides and proteins, the polynucleotides (DNA and RNA), and the polysaccharides. The paper presented here will deal mainly with the determination of the three-dimensional structure of proteins although the same methods can be applied to polynucleotides and polysaccharides as well [3–8].

2. Motivation: why do we need structure determination by NMR?

X-ray diffraction represents a well-established method for the determination of the three-dimensional structure of proteins, in fact almost all of our present knowledge about the structure–function relation is based on X-ray structures and existing theories are developed in

conjunction with these structures. There are now more than 400 protein structures solved. When one succeeds in growing appropriate protein crystals, the three-dimensional structure is obtained more or less routinely (for recent reviews see refs. [9, 10]).

Why do we need NMR structures? There are several good reasons for it; first of all, NMR is not dependent on single crystals of proteins, the method described in the following is suitable for dissolved proteins. In fact, crystals of proteins would require different NMR methods (solid state NMR) and strategies. Since attempts to crystallize proteins often last for many years. NMR can provide the three-dimensional structure of proteins more directly and more rapidly. But even if crystals are available and the crystal structure has been solved, it could be worthwhile to solve the structure also by NMR. Most proteins, especially enzymes, are working in the solution state and only their structure in solution determines the biological function. Before NMR there was no method to determine the structure in solution and it has been assumed that solution structure and crystal structure are essentially identical. After a while this assumption has been generally accepted, although the correct folding of the protein is known to depend on the interaction with the solvent, slight changes in solvent characteristics, e.g. changes in pH or ionic strength can lead to the unfolding (denaturation) of a protein. In crystals these interactions are modified (although protein crystals contain a rather large quantity of water, usually between 30 and 70%) and are partially replaced by protein–protein interactions in the lattice.

3. Structure determination of proteins by NMR

The general strategy for the determination of the three-dimensional structure of proteins in solution was developed by Wüthrich and coworkers in 1982 [11, 12] in close cooperation with Ernst. It still applies in its fundamental ideas, even if the method has been improved by many experimental and computational details [3–10]. Although NMR experiments can be performed with any nucleus with a nuclear spin greater than 0, structure determination by NMR is usually ^{1}H NMR since the ^{1}H nucleus has the highest NMR sensitivity (except for the radioactive isotope of hydrogen ^{3}H) and since hydrogen is abundant in proteins. Other nuclei of potential and probably increasing interest appearing in proteins are nitrogen and carbon with their spin $\frac{1}{2}$ isotopes ^{15}N and ^{13}C.

The main source of information about the spatial relations in a protein molecule arises from the dipolar interaction between nuclear spins that are dependent on the mutual interatomic (interspin) distance r_{ij}. Its influence can be measured as the nuclear Overhauser effect (NOE) (see e.g. refs. [3, 13–15]). For small mixing times τ_m (see below) the NOE caused by interaction of the nuclear spins of atoms i and j is given by

$$\mathrm{NOE}_{ij} \propto r_{ij}^{-6} \tau_m \qquad (1)$$

By comparison with the NOE caused by two spins in a known, fixed distance, the unknown interatomic distances between the two atoms i and j in a macromolecule can be determined.

The second structural information comes from the J-coupling, an interaction between two nuclear spins transferred by the electron spins of the intervening bonds. The magnitude of the J-coupling is dependent on the dihedral (torsional) angles θ. It follows the empirical Karplus equation [16]

$$J(\theta) = A \cos^2\theta - B \cos\theta + C \qquad (2)$$

where A, B, C are constants which are usually estimated from model compounds [17–19].

There are several other sources which contain structural information such as changes of chemical shifts and exchange rates of amide protons, but in general they cannot be interpreted quantitatively.

With the usually known chemical structure of the protein defined by its amino acid sequence and the covalent structures of the different amino acid residues one calculates three-dimensional structures consistent with the distance and dihedral angle constraints (fig. 2).

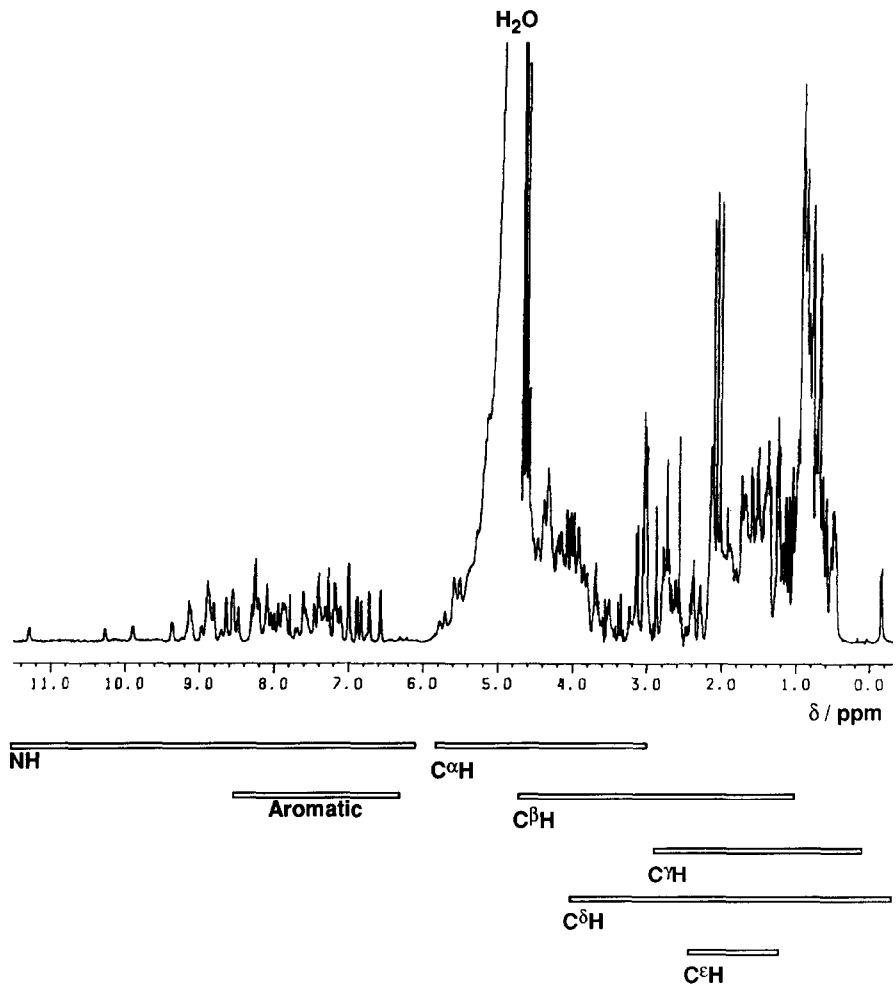

Fig. 1. 500 MHz ^{1}H NMR spectrum of HPr-protein from *S. aureus* in aqueous solution. Approximately 2.5 μmol of protein, total acquisition time 6 min, attenuation of the water signal by presaturation.

Since a protein contains a large number of hydrogen nuclei in chemically inequivalent positions, its ^{1}H NMR spectrum consists of a superposition of a large number of resonance lines. Figure 1 shows the NMR spectrum of a small protein, the HPr protein from *Staphylococcus aureus*. It is composed of 87 amino acids that contain 585 protons whose resonance lines add up to the spectrum shown in fig. 1.

The measurement of the pairwise interatomic distances by NOEs as well as the determination of dihedral angles by *J*-couplings is rather useless if it is unknown which protons in the macromolecule they belong to. Therefore, the resonance lines must first be assigned to specific atoms in the molecule. Although the resonance positions (chemical shifts) are spread over approximately 12 ppm in ^{1}H NMR spectra, neither all resonance lines can be resolved in the one-dimensional NMR spectrum nor can they be assigned individually in such a spectrum. This can only be done by two-dimensional NMR spectroscopy. A set of different 2D spectra must be recorded and evaluated. After assigning all (or at

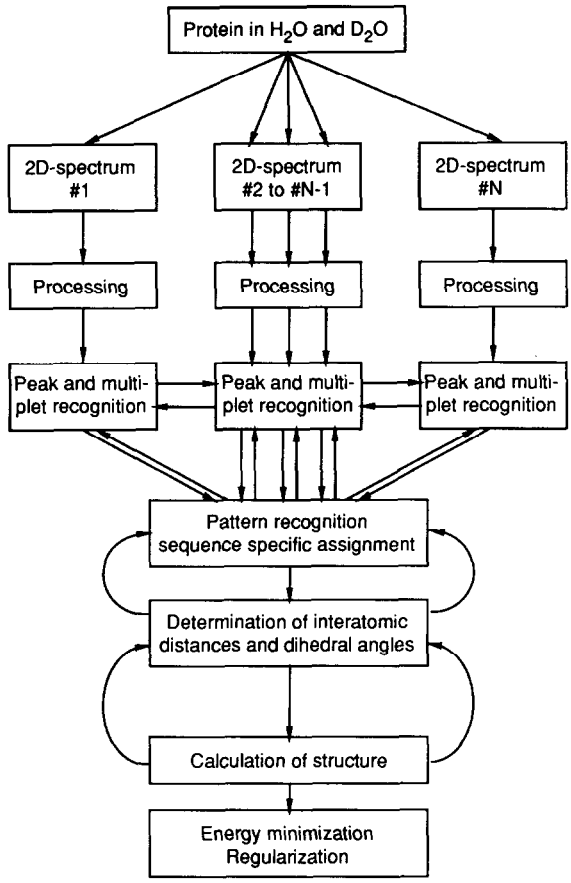

Fig. 2. Schematic representation of structure determination by NMR.

least most) resonances, NOEs and coupling constants J are extracted from the 2D-spectra and the structure is calculated (fig. 2).

4. Two-dimensional NMR spectroscopy

Two-dimensional NMR spectroscopy first proposed by Jeener [20] represents probably one of the most important recent developments in nuclear magnetic resonance; without two-dimensional methods the structure determination of proteins by NMR would be impossible [3, 13]. Approximately 500 different experimental schemes for 2D NMR are published and the number of proposed 2D experiments is still increasing. Moreover, the generalization of the idea of 2D NMR to three-dimensions simply by combining any of the known 2D NMR experiments has increased the number of conceivable experiments further [21–34]. Fortunately, only a small number of basic 2D experiments (and in future probably a few 3D experiments) are necessary for structure determination of proteins. These are the DQF-COSY (double quantum filtered correlated spectroscopy) experiments [35], the RCT (relayed coherence transfer) experiments [36], the NOESY (nuclear Overhauser enhancement spectroscopy) experiments [37] (fig. 3) and the TOCSY (total correlated spectroscopy) experiments [38]. The typical 2D experiment consists of four phases, the preparation phase (often only a single 90° radio frequency pulse), the variable evolution phase t_1, the mixing phase t_m, and the detection phase t_2. During the detection time the NMR signal is sampled as in a 1D experiment and the data are stored. The evolution time t_1 is increased from experiment to experiment according to the Nyquist sampling theorem, the data obtained are stored as a 2D time domain matrix, and after a 2D Fourier transformation a 2D spectrum in the frequency domain is obtained.

What are the advantages of the 2D NMR spectroscopy? First of all, the resolution is enhanced dramatically, since the information about the spin system principally contained in the 1D

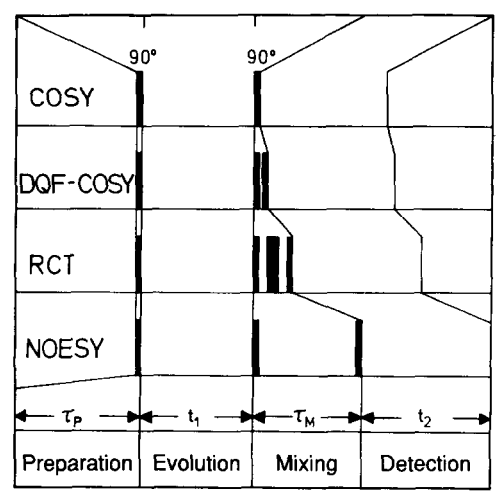

Fig. 3. The two-dimensional NMR experiment.

spectrum is now distributed on two dimensions. Furthermore, depending on the pulse sequence used it is possible to select for distinct properties of the spin system, e.g. for NOEs or J-couplings.

5. Assignments of resonance lines

Figure 4 shows a small part of the amino acid sequence of the HPr protein from *Staphylococcus aureus* containing the amino acid alanine (Ala), aspartic acid (Asp) and glycine (Gly). Observable J-couplings are indicated by broken lines, usually observed NOEs between neighbouring amino acids. Since the magnitude of the J-coupling decreases with the number of intervening bonds, proton–proton coupling can usually not be observed if the nuclei are separated by more than three bonds, that is the J-coupling is not observed between neighbouring amino acids separated by the peptide bond, therefore the spin systems of the individual amino acids are isolated from each other. It is clear that each type of amino acid has a characteristic coupling pattern depending on its chemical structure, resonances belonging to a given amino acid residue can be recognized by their mutual J-coupling.

These coupling patterns can be followed especially well in two-dimensional correlated NMR spectra. Figure 5 shows schematically what is to be expected for the amino acid valine (Val). In a COSY spectrum the information content of the peaks located on the diagonal corresponds essentially to that of an ID spectrum, that is the diagonal peaks give the resonance positions of the individual protons, in our case of the amide, α-, β- and γ-protons. More important are the so-called cross peaks, they show which protons are mutually coupled; that is the amide proton is coupled to the α-proton, the α-proton to the β-proton and the β-proton to the protons of the two methyl groups. In the RCT spectrum the cross peaks reflect the couplings of two resonances via a common coupling partner, so in our example they show the coupling between the amide proton spin transferred via the H_α spin to the H_β spin, the coupling between H_α and the methyl spins transferred via the H_β spin, and the coupling between H_α and the methyl groups transferred via the H_β spin (fig. 5). These coupling patterns have to be identified in 2D spectra of polypeptides which consist of a multitude of cross peaks from a large number of amino acid residues. Figure 6 shows a DQF-COSY spectrum of HPr proteins where the coupling patterns of

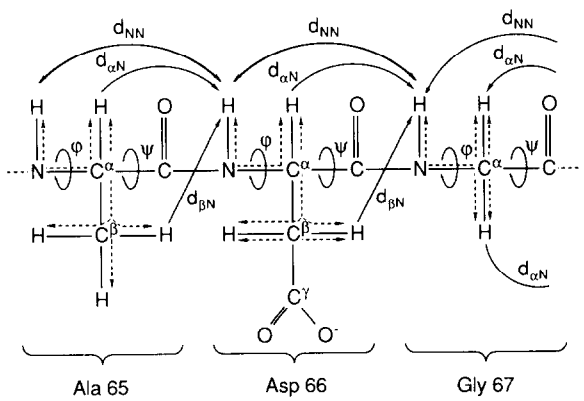

Fig. 4. J-couplings and NOEs in a polypeptide chain. (← - - - →) observable J-coupling between protons, (⟷) NOEs between neighbouring amino acid residues. The J-coupling between the protons of the methyl group is usually not observable, because the protons are equivalent by fast thermally activated rotation.

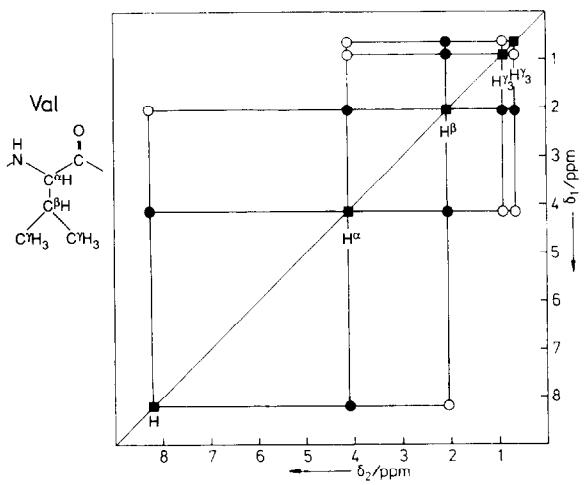

Fig. 5. Schematic view of a coupling pattern in a DQF-COSY and RCT spectrum of a valine residue. (□) diagonal peaks, (●) cross peaks in a DQF-COSY and (○) cross peaks in a RCT spectrum of valine. The additional multiplet structure of the diagonal and cross peaks are not represented.

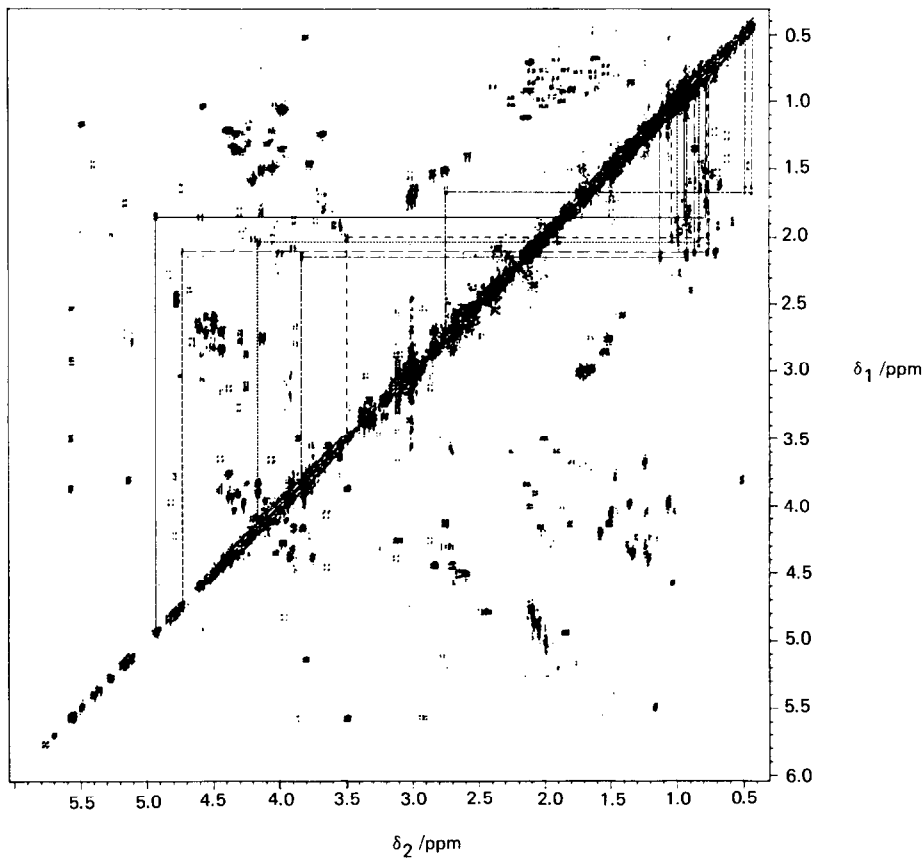

Fig. 6. The valine spin systems of HPr protein. 500 MHz DQF-spectrum of HPr protein from *S. aureus* dissolved in D_2O where the exchangeable amide protons are replaced by deuterons and are not visible in the proton NMR spectrum. The valine spin systems are indicated.

its six valine residues are indicated. In crowded and overlapping spectra it is often useful to refer to an additional experiment, the TOCSY experiment, which shows also cross peaks between spins which are coupled indirectly via a larger number of intervening *J*-coupled spins, which means in our example (fig. 5) cross peaks between the amide and methyl protons of valine would be observable.

Since in a protein usually any type of the 20 directly DNA coded amino acids occur several times, knowledge of the position of a spin system in the amino acid sequence is necessary for a complete assignment. This cannot be done by *J*-coupling because there is no *J*-coupling between neighbouring amino acids (fig. 4). Here, one has to resume to the nuclear Overhauser effect measured in the NOESY experiment since the distance between protons in neighbouring amino acids is limited by the chemical structure. Usually one observes at least one sequential NOE (fig. 4), since at least one of distances d_{NN}, $d_{\alpha N}$ or $d_{\beta N}$ is always shorter than 0.5 nm, the practical upper limit for the observation of NOEs. With these NOEs one can go from spin system to spin system in the sequence. One difficulty is the fact that additional NOEs usually exist between amino acids that are nearby in the three-dimensional structure, but that are not neighbours in the sequence. This problem can

usually be solved by comparison of the assigned spin systems to the spin systems expected from the always known amino acid sequence.

6. Computer aided evaluation of data

It is clear that the evaluation of 2D NMR spectra of proteins which contain a large number of cross peaks (a COSY spectrum of the already mentioned HPr protein should contain approximately 1200 cross peaks splitted again in multiplets with a complex fine structure) can be complicated, tedious and time consuming. Therefore, many attempts were undertaken to improve and speed up the data evaluation by computerizing parts of the procedure.

There are three different levels where computer aid could be very useful: (1) the raw data can be improved by suitable processing of the time domain or frequency domain data, (2) the peak and multiplet identification can be automated, and (3) the recognition and assignment of the spin systems as well as the extraction of the NOE information can be supported by suitable procedures.

Improvement of time domain data by filtering and removal of DC-levels of the signal is standard for long times, corresponding procedures are part of any commercial NMR software package. In the frequency domain a number of procedures are proposed for removing artifacts in the spectra or increasing the signal-to-noise ratio [39–54]. Correct application of these procedures can make the extraction of information much easier and enhance the capability of programs for computer aided pattern recognition.

The effectivity of peak and multiplet recognition depends critically on the a priori information about the peak and multiplet structure expected in a given type of 2D spectrum, therefore a number of different procedures have been published [49, 55–70] that are often only suited for a special type of a 2D experiment.

A rather general procedure we favour [71] uses well-known methods of image analysis: after segmentation and extraction of objects, the objects are clustered and interpreted. In a 2D spectrum the basic objects are the resonance peaks, a typical 2D-spectrum of a protein contains at least 10 000 peaks including peaks of artifacts and noise. These peaks are clustered

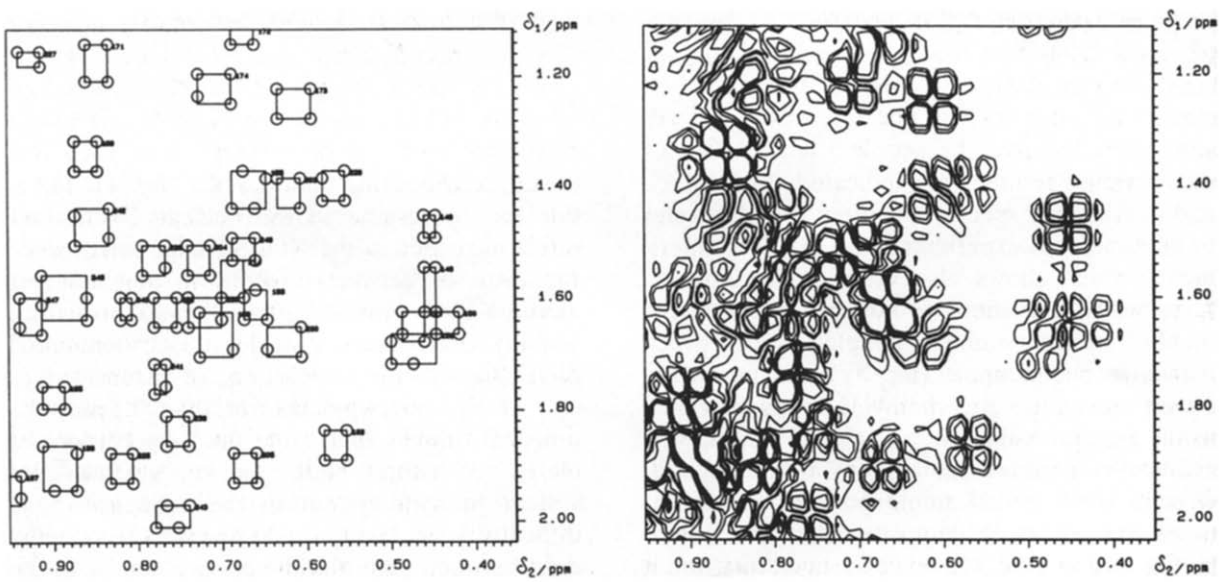

Fig. 7. Multiplet recognition. Small part of a 500 MHz DQF-spectrum of HPr protein of *S. aureus* dissolved in H_2O. The original spectrum (right) and the found multiplets (left) are depicted.

according to their intensity and to their mutual distance. Interpretation of the clustered objects, that is recognition of multiplets, is done by comparison to user defined theoretical multiplet patterns. The center of gravity of test patterns and theoretical patterns is positioned at the origin of a common coordinate system. After rescaling to identical radii of gyration, the individual peaks in test and theoretical pattern are mutually assigned by their minimum distances, and a correlation factor is calculated by matching shapes, intensity and radial angle distribution. If the match of these features is sufficient, a multiplet is accepted as recognized. Figure 7 shows an example of such a multiplet search in a DQF-COSY of HPr protein. Most peaks are recognized correctly, but as was to be expected some multiplets would be interpreted differently from a human observer which usually disposes over additional non-local information for the interpretation of data. Therefore, any such procedure should be interactively controlled by the user which can make a decision in unclear cases.

The recognition of spin systems is the next important step. Although many different methods were proposed [55, 58, 60, 68–69, 72–79] and work rather well in spectra of oligopeptides or for limited problems, no satisfactory program exists up to this moment. But our experience shows that their design has to have at least two characteristics, it has to use the information of different 2D spectra simultaneously and it has to solve the problem iteratively and user supported.

7. Calculation of the three-dimensional structure

In the beginning, the calculation of three-dimensional structures from NMR data appeared to be a major problem, but with the increasing availability of powerful computers and the development of commercial program packages containing the most efficient algorithms these calculations can be done almost routinely. Although it is still discussed sometimes rather emotionally that the just favoured algorithm is superior to all others because it uses less information (e.g. only information from NMR data) and therefore should be unbiased, it is generally realized that the primary goal is to find a solution (or the solutions) consistent with all available knowledge about the system as efficiently as possible. In fact, all programs discussed refer to the chemical structure of the macromolecule in some way. A different question is how meaningful is the calculated structure. Here, we have two difficulties, the general problem how to define the term structure of a macromolecule in a solute state and the more mathematical problem, how to determine the accuracy and uniqueness of a solution if a generally accepted definition of the structure should exist.

Side chains on the exterior, but often also in the interior of proteins are highly mobile, and even in the polypeptide backbone large scale cooperative movements can be expected. Moreover, enzymatic catalysis by proteins often requires transitions between a number of different conformational states which may coexist in solution. Apparently, taking the time and the ensemble average in an appropriate internal coordinate system and arguing with this average structure of the macromolecule can be completely misleading, since the average between different possible structures does not necessarily lead to a structure which is energetically possible. One solution would be taking the most probable (that is the structure with the lowest energy in the ensemble) as the structure of the protein. But even this definition has its disadvantages since it is not a priori clear and depends probably on the protein studied if the native (biologically functional) conformation of a protein is the conformation with the lowest energy. The native conformation could only represent a local energy minimum of the system which is only populated transiently and which is depopulated with time (denaturation) or it could be located near (in energy) to the lowest energy state and be populated by thermal activation. Moreover, the experimental data itself are time- and ensemble averages, which are weighed non-linearily.

In spite of all these difficulties the best one can probably do is trying to calculate a number

representative structures consistent with the experimental data and compare them.

Historically, two main algorithms were used for this aim, the distance algorithm and the restrained molecular dynamics (for recent reviews see refs. [5, 80–81]).

The distance algorithm [80–90] represents a rather effective way to search the conformational space for solutions. The basic idea is to express all conditions on a solution as distance constraints and perform an optimization in the Cartesian space. The macromolecule itself is essentially described by a hard spere model, Van der Waals radii, bond lengths and bond angles as well as distance limits obtained from NOE measurements can rather naturally be expressed by upper and lower distance limits U_{ij} and L_{ij} between the atoms i and j. It is somewhat more difficult to code chiralities and constraints on dihedral angles as distance limits between atoms. The structures obtained have usually a distorted geometry and have to be regularized which is often done by energy minimization.

Restrained molecular dynamics simulates the macromolecule by numerically integrating Newton's equations of motion [91–101]

$$m_i \mathrm{d}r_i/\mathrm{d}t_i = -V \,. \tag{3}$$

Experimental constraints are usually added to the intramolecular physical potential V as pseudo potentials, a typical simple pseudo potential V_{dc} for the distance constraint $d_{ij,0}$ (most probable distance between atom i and j) is given by

$$V_{\mathrm{dc}} = \tfrac{1}{2} K_{\mathrm{dc}} (d_{ij} - d_{ij,0})^2 \tag{4}$$

where K_{dc} is a constant whose value determines the weight of the pseudo potential relative to the physical potential. After a number of integration steps one has to equilibrate the total kinetic energy with the temperature bath; approximating the energy minimum means an increase in kinetic energy, that is, one has to cool the molecule for stabilizing the result. When one wishes to come out of a local minimum one can heat up the molecule for a short time, that is, increase the kinetic energy of all degrees of freedom of the macromolecule. Again restrained molecular dynamics can be seen in different ways, either as calculation of a structure from general physical knowledge about the relevant potentials guided by additional experimental data or as an effective search procedure for an optimal solution which is consistent with the experimental data. The integration of the equation of motion would now represent a special optimization procedure, the additional physical potentials would help to guide the search. With the second point of view one is free to add, remove or modify the expression for the potential energy function, as long as the convergence according to the experimental constraints is granted. This has been done in e.g. refs. [97, 100, 101].

8. Discussion

It is clear that the determination of the three-dimensional structure of small, soluble proteins by NMR is now a well-established method which will be more and more important in the future. The structures obtained are often very similar to the structures obtained by X-ray crystallography, the model case is the structure of the α-amylase inhibitor tendamistat whose X-ray and NMR structure was determined independently by two different groups with high resolution. Only minor differences of side chain conformations of surface residues could be observed [102–104]. In fact, in the beginning nobody would have accepted NMR as a structural method, if NMR and crystal structure would have differed in important details. The situation has changed much since then and more and more examples are published where the solution structure is quite different. Two instructive examples are the metal binding protein metallothionein and the phosphorcarrier protein HPr. In metallothionein, the metal coordination and the groups participating in the metal binding are different in solution and in crystalline form [105–108]. In the HPr protein from *E. coli* the tertiary structure in solution and in the crystal are completely different, for example a four-stranded β-pleated sheet existing in the solution does not exist in the X-ray structure

[109–114]. The existence of this folding pattern in the solution has now been established by NMR for two different, homologous HPr proteins, HPr from *S. faecalis* [114] and from *S. aureus* (fig. 8).

Does this mean that the X-ray structures are wrong? Most probably not, but it means that in these cases the structures determined by X-ray crystallography do not represent the conformation which is dominant in a solution. The number of NMR structures showing significant differences to X-ray structures is increasing, which means that although often correct, one can never be sure that the crystal structure obtained is a good model for the solution structure. In this respect, an interesting example has been published recently: for a long time biochemical as well as spectroscopic evidence postulated a hydrogen bridge between a histidine and a serine residue in the active center of the serine proteases, but this was in conflict with the published X-ray structure where such an arrangement was not observable. A recent NMR study [115] could prove that a minor rearrangement of the active center structure, probably induced by a change in pK of the active center histidine by a sulfate ion present in the crystal, caused this obvious discrepancy.

Since NMR is and will be limited to rather small proteins, a main advantage of X-ray crystallography still remains: the molecular weight of the protein is no severe limitation as long as good crystals are available, whereas for NMR very high molecular weights (e.g. 100 000 d) are prohibitive. Today, the structure of proteins with 100 amino acids can be solved routinely, a limit which can probably be shifted to 200 amino acids in the near future by making already existing methods more perfect. With isotope labelling, 3D spectroscopy, site directed mutagenesis and higher magnetic fields one will probably come to somewhat higher molecular weights in future.

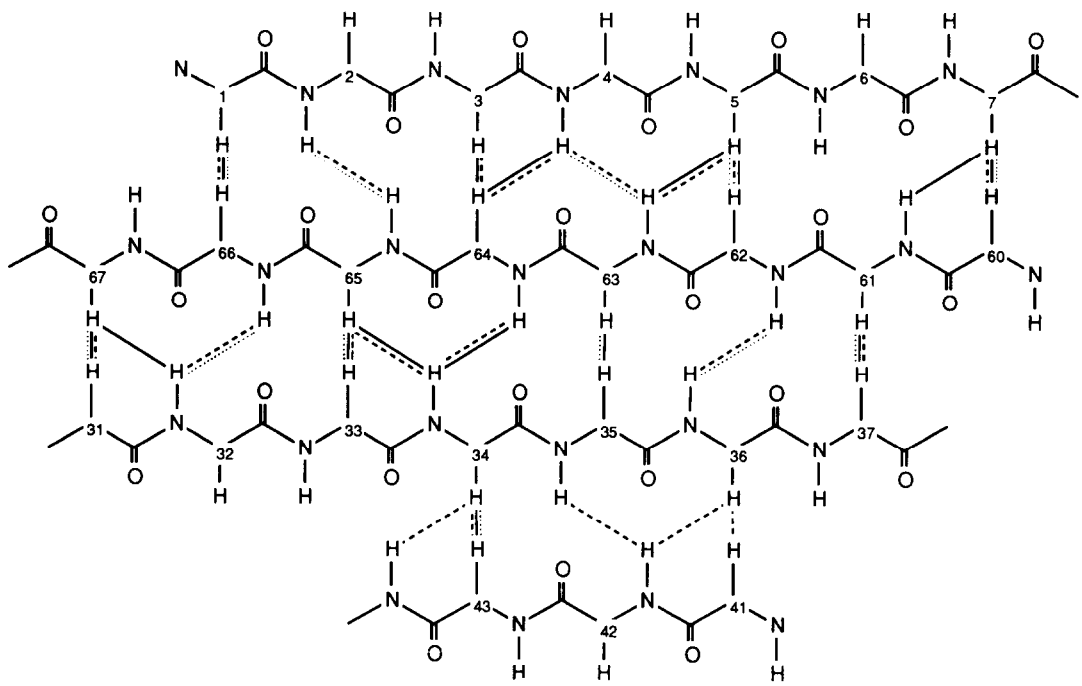

Fig. 8. β-pleated sheet in HPr protein from *S. aureus*. The interchain NOEs observed for HPr protein from *S. aureus* (———), *S. faecalis* (-------) [114], and *E. coli* (– – –) [112] are depicted.

References

[1] K. Wüthrich, NMR in Biological Research: Peptides and Proteins (North-Holland, Amsterdam, 1976).
[2] O. Jardetzky and G.C.K. Roberts, NMR in Molecular Biology (Academic, New York, 1981).
[3] K. Wüthrich, NMR of Proteins and Nucleic Acids (John Wiley, New York, 1986).
[4] K.H. Hausser and H.R. Kalbitzer, NMR in Medicine and Biology, Structure Determination, Tomography, In Vivo Spectroscopy (Springer, Heidelberg, 1990).
[5] R. Kaptein, R. Boelens, R.M. Scheek and W.F. van Gunsteren, Biochemistry 27 (1988) 5389.
[6] K. Wüthrich, Acc. Chem. Res. 22 (1989) 36.
[7] P.E. Wright, TIBS 14 (1989) 255.
[8] A. Bax, Ann. Rev. Biochem. 58 (1989) 223.
[9] H.W. Wyckoff, C.H.W. Hirs and S.N. Timasheff, eds. Methods in Enzymology, Vols. 114 & 115 (Academic, New York, 1985).
[10] D. Eisenberg and C.P. Hill, TIBS 14 (1989) 260.
[11] K. Wüthrich, G. Wider, G. Wagner and W. Braun, J. Mol. Biol. 155 (1982) 311.
[12] G. Wagner and K. Wüthrich, J. Mol. Biol. 155 (1982) 347.
[13] R.R. Ernst, G. Bodenhausen and A. Wokaun, Principles of Nuclear Magnetic Resonance in One and Two Dimensions (Clarendon, Oxford, 1987).
[14] J.H. Noggle and R.E. Schirmer, The Nuclear Overhauser Effect, Chemical Applications (Academic, New York, 1971).
[15] D. Neuhaus and M.P. Williamson, The Nuclear Overhauser Effect in Structural and Conformational Analysis (VCH, New York, 1989).
[16] M. Karplus, J. Chem. Phys. 30 (1959) 11.
[17] A. de Marco, M. Llinås and K. Wüthrich, Biopolymers 17 (1978) 617.
[18] A. de Marco, M. Llinås and K. Wüthrich, Biopolymers 17 (1978) 637.
[19] A. Pardi, M. Billeter and K. Wüthrich, J. Mol. Biol. 180 (1984) 741.
[20] J. Jeener, Ampère Summer School in Basko Polje (1971).
[21] H.D. Plant, T.H. Mareci, M.D. Cockman and W.S. Brey, 27th Experimental NMR Conf., Baltimore (1986).
[22] G.M. Vuister and R. Boelens, J. Magn. Reson. 73 (1987) 328.
[23] C. Griesinger, O.W. Sørensen and R.R. Ernst, J. Magn. Reson. 73 (1987) 574.
[24] C. Griesinger, O.W. Sørensen and R.R. Ernst, J. Am. Chem. Soc. 109 (1987) 7227.
[25] R.E. Hoffman and D.B. Davies, J. Magn. Reson. 80 (1988) 337.
[26] G.W. Vuister, R. Boelens and R. Kaptein, J. Magn. Reson. 80 (1988) 176.
[27] H. Oschkinat, C. Griesinger, P.J. Kraulis, O.W. Sørensen, R.R. Ernst, A.M. Gronenborn and G.M. Clore, Nature 332 (1988) 374.
[28] S.W. Fesik and E.R.P. Zuiderweg, J. Magn. Reson. 78 (1988) 588.
[29] C. Griesinger, O.W. Sørensen and R.R. Ernst, J. Magn. Reson. 84 (1989) 14.
[30] S.W. Fesik, R.T. Gampe, Jr., E.R.P. Zuiderweg, W.E. Kohlbrenner and D. Weigl, Biochem. Res. Comm. 159 (1989) 842.
[31] D. Marion, L.E. Kay, S.W. Sparks, D.A. Torchia and A. Bax, J. Am. Chem. Soc. 111 (1989) 1515.
[32] D. Marion, P.C. Driscoll, L.E. Kay, P.T. Wingfield, A. Bax, A.M. Gronenborn and G.M. Clore, Biochemistry 28 (1989) 6150.
[33] L.E. Kay, D. Marion and A. Bax, J. Magn. Reson. 84 (1989) 72.
[34] S.S. Wijmenga, K. Hallenga and C.W. Hilbers, J. Magn. Reson. 84 (1989) 634.
[35] M. Rance, O.W. Sorensen, G. Bodenhausen, G. Wagner, R.R. Ernst and K. Wüthrich, Biochem. Biophys. Res. Comm. 117 (1983) 479.
[36] G. Eich, G. Bodenhausen and R.R. Ernst, J. Am. Chem. Soc. 104 (1982) 3731.
[37] J. Jeener, B.H. Meier, P. Bachmann and R.R. Ernst, J. Chem. Phys. 71 (1979) 4546.
[38] L. Braunschweiler and R.R. Ernst, J. Magn. Reson. 53 (1983) 521.
[39] R. Baumann, A. Kumar, R.R. Ernst and K. Wüthrich, J. Magn. Reson. 44 (1981) 76.
[40] R. Baumann, G. Wider, R.R. Ernst and K. Wüthrich, J. Magn. Reson. 44 (1981) 402.
[41] R.E. Klevit, J. Magn. Reson. 62 (1985) 551.
[42] P.H. Bolton, J. Magn. Reson. 64 (1985) 352.
[43] S. Glaser and H.R. Kalbitzer, J. Magn. Reson. 68 (1986) 350.
[44] Z. Zolnay, S. Macura and J.L. Markley, Comput. Enhanced Spect. 3 (1986) 141.
[45] P.H. Bolton, J. Magn. Reson. 67 (1986) 391.
[46] P.H. Bolton, J. Magn. Reson. 68 (1986) 180.
[47] P.H. Bolton, J. Magn. Reson. 77 (1986) 344.
[48] I.L. Barsukov and A.S. Arseniev, J. Magn. Reson. 73 (1987) 148.
[49] J.C. Hoch, S. Hengyi, M. Kjaer, S. Ludvigsen and F.M. Poulsen, Carlberg Res. Comm. 52 (1987) 111.
[50] K.-P. Neidig and H.R. Kalbitzer, Magn. Reson. Chem. 26 (1988) 848.
[51] Z. Zolnay, S. Macura and J.L. Markley, J. Magn. Reson. 80 (1988) 60.
[52] P. Solé, F. Delaglio and G.C. Levy, J. Magn. Reson. 80 (1988) 517.
[53] Z. Zolnay, S. Macura and J. Markley, J. Magn. Reson. 82 (1989) 496.
[54] K.-P. Neidig and H.R. Kalbitzer, J. Magn. Reson. (1990) in press.
[55] K.-P. Neidig, H. Bodenmüller and H.R. Kalbitzer, Biochem. Biophys. Res. Comm. 125 (1984) 1143.

[56] B.U. Meier, G. Bodenhausen and R.R. Ernst, J. Magn. Reson. 60 (1984) 161.
[57] P. Pfändler, G. Bodenhausen, B.U. Meier and R.R. Ernst, Anal. Chem. 57 (1985) 2510.
[58] P. Pfändler and G. Bodenhausen, J. Magn. Reson. 70 (1986) 71.
[59] G. Bodenhausen, J. Mol. Struct. 141 (1986) 255.
[60] B.U. Meier, Z.L. Mådi and R.R. Ernst, J. Magn. Reson. 74 (1987) 565.
[61] M. Novic, H. Oschkinat, P. Pfändler and G. Bodenhausen, J. Magn. Reson. 73 (1987) 493.
[62] Z. Mådi, B.U. Meier and R.R. Ernst, J. Magn. Reson. 72 (1987) 584.
[63] S. Glaser and H.R. Kalbitzer, J. Magn. Reson. 74 (1987) 450.
[64] U. Eggenberger, P. Pfändler and G. Bodenhausen, J. Magn. Reson. 77 (1988) 192.
[65] M. Novic, U. Eggenberger and G. Bodenhausen, J. Magn. Reson. 77 (1988) 394.
[66] B.U. Meier and R.R. Ernst, J. Magn. Reson. 79 (1988) 540.
[67] M. Novic and G. Bodenhausen, Anal. Chem. 60 (1988) 582.
[68] P. Pfändler and G. Bodenhausen, Magn. Reson. Chem. 26 (1988) 888.
[69] P. Pfändler and G. Bodenhausen, J. Magn. Reson. 79 (1988) 99.
[70] V. Stoven, A. Mikou, D. Piveteau, E. Guittet and J.-Y. Lallemand, J. Magn. Reson. 82 (1989) 163.
[71] K.-P. Neidig, R. Saffrich, M. Lorenz and H.R. Kalbitzer, J. Magn. Reson. (1990) in press.
[72] S.W. Englander and A.J. Wand, Biochemistry 26 (1987) 5953.
[73] H. Grahn, F. Delaglio, M.A. Delsuc and G.C. Levy, J. Magn. Reson. 77 (1988) 294.
[74] C. Cieslar, G.M. Clore and A.M. Gronenborn, J. Magn. Reson. 80 (1988) 119.
[75] M. Billeter, V.J. Basus and I.D. Kuntz, J. Magn. Reson. 76 (1988) 400.
[76] Z.L. Mådi and R.R. Ernst, J. Magn. Reson. 79 (1988) 513.
[77] P. Günert, W. Braun, M. Billeter and K. Wüthrich, J. Am. Chem. Soc. 111 (1989) 3997.
[78] C.D. Eads and I.D. Kuntz, J. Magn. Reson. 82 (1989) 467.
[79] P.J. Kraulis, J. Magn. Reson. 84 (1989) 627.
[80] W. Braun, Quart. Rev. Biophys. 19 (1987) 115.
[81] G.M. Clore and A.M. Gronenborn, Prot. Engineering 1 (1987) 275.
[82] L.M. Blumenthal, Theory and Applications of Distance Geometry (Chelsea, New York, 1970).
[83] G.M. Crippen, J. Comp. Phys. 26 (1977) 449.
[84] G.M. Crippen and T.F. Havel, Acta Crystallogr. A 34 (1981) 282.
[85] W. Braun, C. Bösch, L.R. Brown, N. Go and K. Wüthrich, Biochim. Biophys. Acta 667 (1981) 377.
[86] T.F. Havel, I.W. Kuntz and G.M. Crippen, Bull. Math. Biol. 45 (1983) 45.
[87] T.F. Havel and K. Wüthrich, Bull. Math. Biol. 46 (1984) 673.
[88] T.F. Havel and K. Wüthrich, J. Mol. Biol. 182 (1985) 281.
[89] W. Braun and N. Go, J. Mol. Biol. 186 (1985) 611.
[90] G. Wagner, W. Braun, T.F. Havel, T. Schaumann, N. Go and K. Wüthrich, J. Mol. Biol. 196 (1987) 611.
[91] D. Beeman, J. Comp. Phys. 20 (1976) 130.
[92] W.F. van Gunsteren and H.J.C. Berendsen, Biochem. Soc. Trans. 10 (1982) 301.
[93] B.R. Brooks, R.E. Bruccoleri, B.D. Olafson, D.J. States, S. Swaminathan and M. Karplus, J. Comp. Chem. 4 (1983) 187.
[94] M. Levitt, J. Mol. Biol. 170 (1983) 723.
[95] R. Kaptein, E.R.P. Zuiderweg, R.M. Scheek, R. Boelens and W.F. van Gunsteren, J. Mol. Biol. 182 (1985) 179.
[96] G.M. Clore, D.K. Sukumaran, M. Nilges and A.M. Gronenborn, J. Mol. Biol. 186 (1985) 435.
[97] A.T. Brünger, G.M. Clore, A.M. Gronenborn and M. Karplus, Proc. Nat. Acad. Sci. USA 83 (1986) 3801.
[98] J. de Vlieg, R. Boelens, R.M. Scheek, R. Kaptein and W.F. van Gunsteren, Isr. J. Chem. 27 (1986) 181.
[99] T.A. Holak, A. Engström, P.J. Kraulis, G. Lindeberg, H. Bennich, T.A. Jones, A.M. Gronenborn and G.M. Clore, Biochemistry 27 (1988) 7620.
[100] R.M. Scheek and R. Kaptein, in: NMR in Enzymology, N.J. Oppenheimer and T.L. James, eds. (Academic, New York, 1988).
[101] T.A. Holak, M. Nilges and H. Oschkinat, FEBS Lett. 242 (1989) 218.
[102] A.D. Kline, W. Braun and K. Wüthrich (1986) J. Mol. Biol. 189 (1986) 377.
[103] J.W. Pflugrath, G. Wiegand, R. Huber and L. Vértesy, J. Mol. Biol. 189 (1986) 741.
[104] M. Billeter, A.D. Kline, W. Braun, R. Huber and K. Wüthrich, J. Mol. Biol. 206 (1989) 677.
[105] M.H. Frey, H. Wagner, M. Vasåk, O.W. Sørensen, D. Neuhaus, E. Wörgötter, J.H.R. Kägi, R.R. Ernst and K. Wüthrich, J. Am. Chem. Soc. 107 (1985) 6847.
[106] M.H. Frey, W. Leupin, O.W. Sørensen, W.A. Denny, R.R. Ernst and K. Wüthrich, Biopolymers 24 (1985) 2371.
[107] W.F. Furey, A.H. Robbins, L.L. Clancy, D.R. Winge, B.C. Wang and C.D. Stout, Science 231 (1986) 704.
[108] W. Braun, G. Wagner, E. Wörgötter, M. Vasåk, J.H.R. Kägi and K. Wüthrich, J. Mol. Biol. 187 (1986) 125.
[109] O.A. El-Kabbani, E.B. Waygood and L.T.J. Delbaere, J. Biol. Chem. 262 (1987) 12962.
[110] R.E. Klevit, G.P. Drobny and E.B. Waygood, Biochemistry 25 (1986) 7760.
[111] R.E. Klevit and G.P. Drobny, Biochemistry 25 (1986) 7770.

[112] R.E. Klevit and E.B. Waygood, Biochemistry 25 (1986) 7774.

[113] E.B. Waygood, S. Sharma, P. Bhanot, O.A.L. El-Kabbani, L.T.J. Delbaerre, F. Georges, M.G. Wittekind and R.E. Klevit, FEMS Microbiol. Rev. 63 (1989) 43.

[114] W. Hengstenberg, B. Reiche, R. Eisermann, R. Fischer, U. Kessler, A. Tarrach, W.M. De Vos, H.R. Kalbitzer and S. Glaser, FEMS Microbiol. Rev. 63 (1989) 35.

[115] O. Smith, S. Farr-Jones, R.G. Griffin and W.W. Bachovchin, Science 244 (1989) 961.