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NMR structures of the micelle-bound polypeptide hormone glucagon†

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The determination of the spatial structure of the polypeptide hormone glucagon bound to perdeuterated dodecylphosphocholine (DPC) micelles was the first attempt to apply two-dimensional (2D) NMR methods to a biologically relevant problem. The glucagon–DPC complex had considerably higher molecular weight than any other molecule investigated by 2D NMR at the time. Nevertheless, almost complete proton resonance assignments could be obtained. The structure determination was not only a challenge for the newly developed 2D NMR methodology, but also for the computers on which the structures were calculated. Micelle-bound glucagon did not adopt a globular tertiary structure; it contained well-defined structured parts, as well as flexible sections. The overall spatial arrangement of the polypeptide chain was largely determined by the topology of the lipid support. The results obtained with the glucagon–DPC system were an essential breakthrough demonstrating the superiority of 2D NMR methods. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: 2D NMR; DPC; resonance assignment; NOESY; distance geometry

INTRODUCTION

In the late 1970s, Kurt Wüthrich gave classes in biophysics at the Eidgenössische Technische Hochschule (ETH) in Zürich. At that time I was a physics student at the ETH attending these classes. In one of his lectures Kurt Wüthrich showed a two-dimensional (2D) *J*-resolved NMR spectrum and in the discussion after the lecture he mentioned the joint project with Richard Ernst in which 2D NMR experiments with proteins should be developed. The technique and its potential promised really exciting developments. Fortunately, Kurt Wüthrich accepted me as a PhD student and in 1978 I started my PhD thesis with the tentative topic '2D NMR with polypeptides and proteins'. The topic was declared tentative because, at that time, it was not clear if the potential of 2D NMR could really be turned into fruitful results with biological macromolecules. 2D NMR of proteins did not turn into a disaster,[1] so I did not have to change topics and received my PhD degree[2] from the ETH based on the referee reports of Kurt Wüthrich and Richard Ernst (Fig. 1). In a few years, 2D NMR had developed from an exotic technique to a broadly accepted method with great experimental potential.[3]

Kurt Wüthrich and Richard Ernst employed extremely talented postdocs in their joint research project on 2D NMR. During my time as a PhD student I had the pleasure to work together with Kuniaki Nagayama, Anil Kumar and Slobodan Macura, who were employed as postdocs within the joint project. I am especially thankful to Kuniaki, who introduced me to the theory and practice of 2D NMR. Theory in those days meant full quantum mechanical treatment; the additional, more intuitive treatments of today were not available. My first project was the investigation of strong coupling in 2D *J*-resolved spectra, which required a full quantum mechanical treatment anyhow.[4] Not only was the available theory less intuitive, but also the practical aspects of 2D NMR on the spectrometer were quite different. Obtaining a 2D spectrum on the spectrometer was not exactly user friendly and needed programming in assembler and using a debugger program to get around hour-long recompilation of the whole source code which would otherwise have been necessary even for a simple change of the phase of a radio-frequency pulse. Pulse programming software, as it is known today, was just in its infancy and far from being capable of permitting programming of 2D NMR experiments. Fortunately, this situation improved gradually, and at the end of my PhD time a limited number of explicitly programmed 2D NMR experiments could be performed on all modern NMR spectrometers.

After the development of COSY,[5] SECSY[6] and NOESY[7] it appeared that the tools were ready for the first complete resonance assignment of a protein by 2D NMR methods.[8] Even the next step could be anticipated: the determination of the three-dimensional structure of a protein based on distance constraints obtained from NOESY spectra. At the time, Werner Braun in our group was working on programs that could calculate three-dimensional structures from NMR data and he was eager to apply his programs to a complete experimental data set. Kurt Wüthrich decided that I should put all the techniques to good use and get at least full

†Dedicated to Professor Kurt Wüthrich on the occasion of his 65th birthday.

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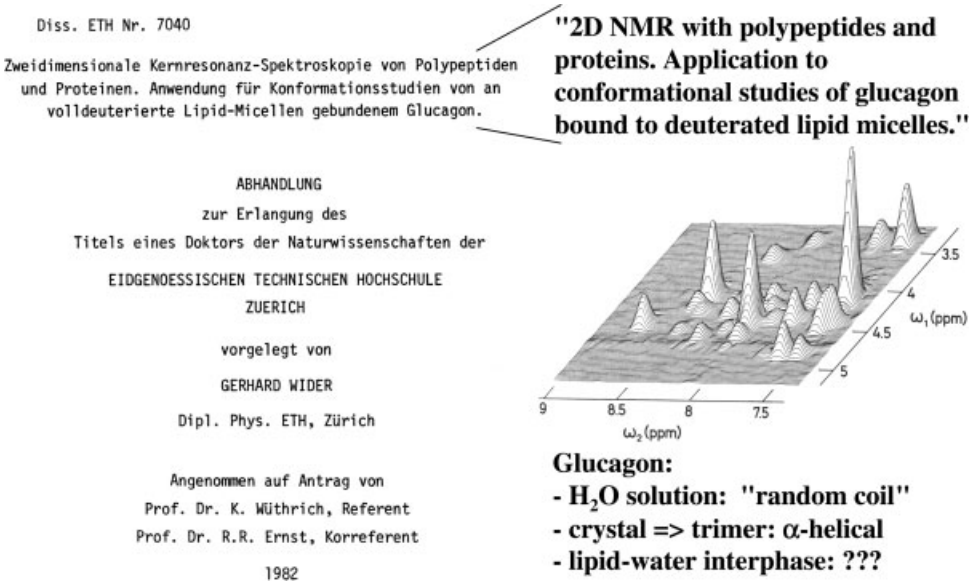

Figure 1. Studies of micelle-bound glucagon.

assignments and possibly a structure of a globular protein as part of my thesis work. The target protein was a sea anemone toxin with 42 amino acids. Based on optical spectroscopy, the protein with three disulfides seemed rather well behaved and the NMR investigation was started. The NMR spectra looked really good, but they contained at least five resonances of ε-protons of histidine instead of the two expected based on the amino acid sequence. No sample conditions could be found that resolved the problem, and the project was shelved because it was not clear how this heterogeneity would affect the assignment process and the structure. Kurt Wüthrich decided that instead of the sea anemone toxin the polypeptide hormone glucagon solubilized in detergent micelles should be studied. This complex had a molecular weight of 17 000 Da, which was far above any other system studied by 2D NMR at that time. Even though glucagon has only 29 amino acid residues, it turned out to be a rather demanding project.

MATERIAL AND METHODS

Glucagon is an important polypeptide hormone and is involved in the activation of glycogenolysis[9] (Fig. 2). It has 29 amino acids residues and a molecular weight of 3 500 Da. The primary target organ for glucagon is the plasma membrane of liver and other cells, where it binds to a specific receptor. There was evidence that recognition between glucagon and its receptor depends on the ordered lipid structures surrounding the receptor site in the membrane. The α-helical conformation found for glucagon trimers in single crystals[10] and the flexible conformation of monomeric glucagon in aqueous solution[11] were considered to be an inadequate description for the interaction between glucagon and its receptor (Fig. 1). It was thus of great interest to investigate interactions of glucagon with lipids and detergents. We used mixed micelles of glucagon and dodecylphosphocholine (DPC). The complex contained one molecule of glucagon and about 40 detergent molecules, which corresponds to

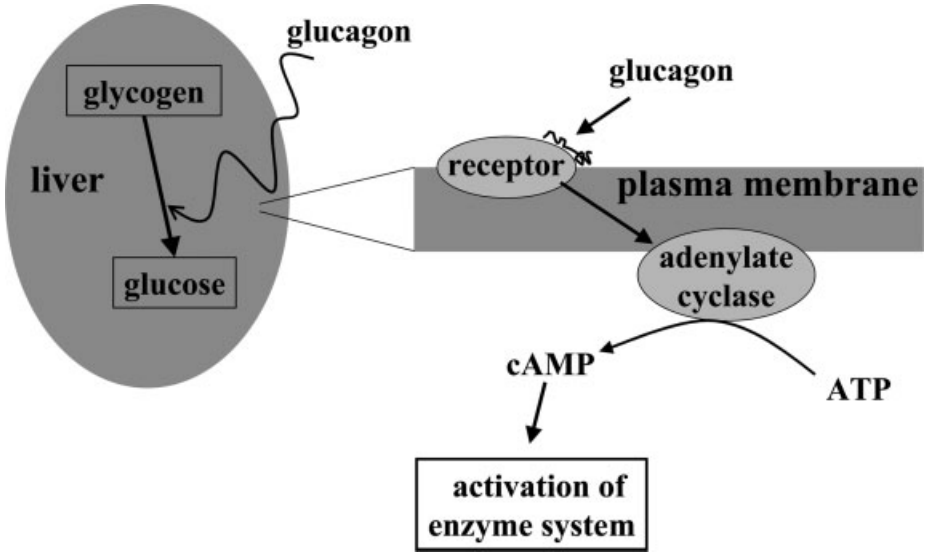

Figure 2. The polypeptide hormone glucagon activates glycogenolysis.

a molecular weight of *ca* 17 000 Da.[11] The sample used for the 2D NMR studies contained 700 mM perdeuterated DPC, 15 mM glucagon, 50 mM phosphate buffer and was measured at pH 6.0 and at a temperature of 37 °C (Fig. 3).

SEQUENTIAL ASSIGNMENT

The complete resonance assignment and the structure determination of glucagon bound to DPC micelles should, for the first time be completely based on homonuclear 2D NMR experiments using COSY (Fig. 1) and NOESY spectra. Since glucagon–DPC had no very slowly exchanging amide protons, unlike the globular proteins studied by 2D NMR in those days, all spectra had to be measured in H_2O solution, which first required a proper implementation of water suppression techniques into the 2D NMR pulse schemes.[12]

Owing to its biological relevance, the project was originally started with 1D ^{1}H NMR; but, despite great efforts, only the spin systems of the non-labile protons of the amino acid residues Ala 19, Val 23 and Trp 25 were identified.[11] In addition, the imidazole resonances of His 1, and the methyl groups of Met 27, Leu 14 and Leu 26 were assigned. With 2D spectra, all non-labile protons and all backbone amide protons were assigned with the exception of H^{γ} of Gln 20 and Gln 24 and the amide protons of His 1 and Ser 2 (Fig. 4). At that time this assignment was an important breakthrough, demonstrating the superiority of 2D NMR methods.[13]

In Fig. 4, the amide region of the ^{1}H spectrum of the glucagon–DPC complex measured in H_2O solution is shown; a three-dimensional view of same spectral region is plotted in Fig. 1. It seems evident from the broad, overlapping resonances that individual amide proton assignments could not be obtained from 1D spectra (Fig. 4). The situation is completely different in the 2D COSY spectrum, where all peaks are resolved and, therefore, could be completely assigned. From today's perspective the COSY spectrum looks

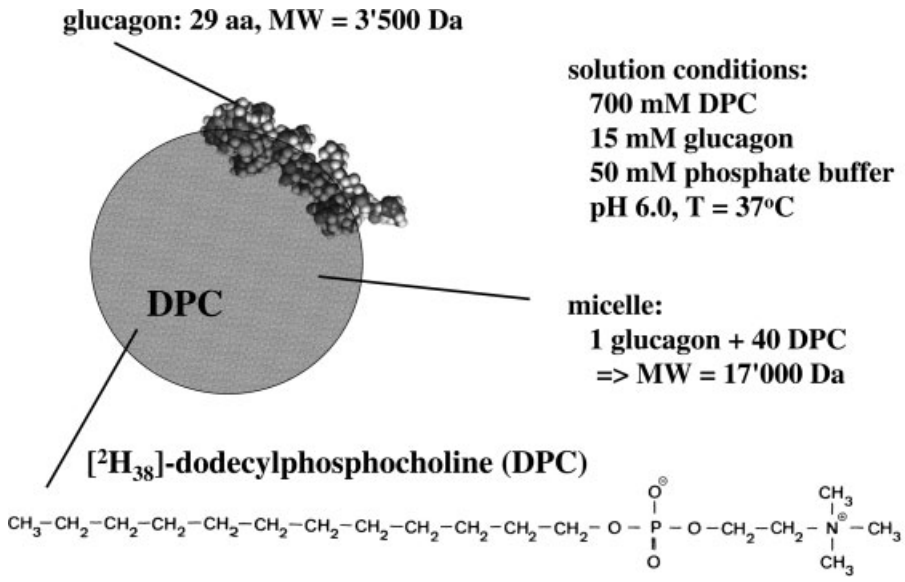

Figure 3. Sample conditions.

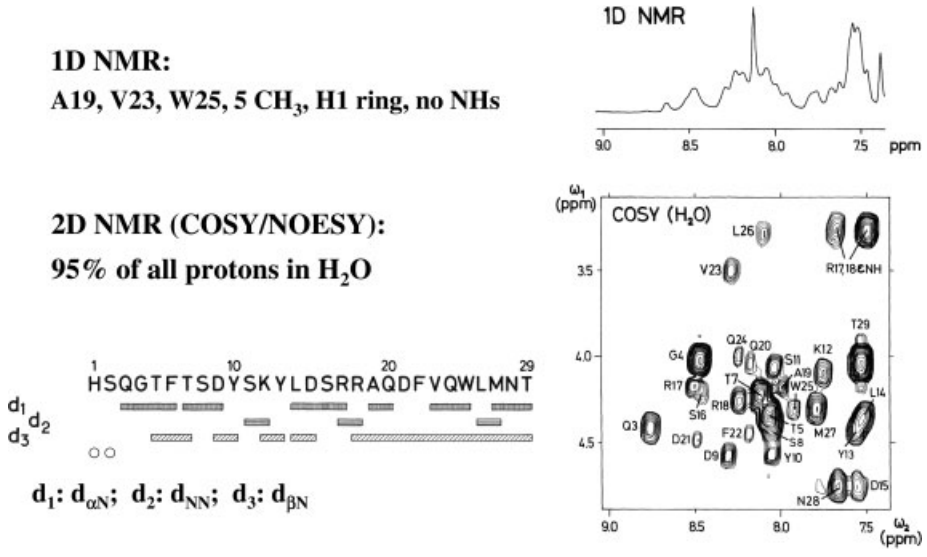

Figure 4. Sequence-specific assignments.

strange, because it is represented in the absolute value mode, as were all spectra in the early days of the development of 2D NMR methods, where phase-sensitive 2D spectra were not available. The inset with the glucagon amino acid sequence in Fig. 4 shows a survey of the experimental data by which individual resonance assignments were obtained for micelle-bound glucagon. The original nomenclature d_1, d_2 and d_3 is used for the sequential connectivities, which has to be translated into the sequential connectivities $d_{\alpha N}$, d_{NN} and $d_{\beta N}$ respectively (Fig. 4).[1] The complete resonance assignment of glucagon bound to DPC micelles was published in the *Journal of Molecular Biology* as a full paper with 21 pages,[13] which compares favorably with the two-page assignment notes of today that are published in the *Journal of Biomolecular NMR*.

When developing the sequential assignment procedure with basic pancreatic typsin inhibitor[14] and glucagon–DPC[13] it became clear that the NOESY sequential connectivities indicate the secondary structure of the polypeptide chain. This information shows for glucagon–DPC that the C-terminal part adopts a helical conformation, whereas the remainder of the polypeptide is more extended with some helix-like turns (Fig. 5). Figure 5 also presents an absolute value NOESY spectrum of the aliphatic region of the glucagon–DPC complex. The spectrum is symmetrized: one of the ancient techniques used to improve the spectral appearance in the early days of 2D NMR with proteins.[15] Nevertheless, the spectrum documents that rather good-quality spectra could be obtained with the glucagon–DPC complex despite the high molecular weight, the absolute value spectra and the residual detergent signals.

STRUCTURE DETERMINATION

The glucagon–DPC complex had a considerably higher molecular weight than any other molecule investigated by 2D NMR at the time, thus it was not clear how much spin diffusion would affect the NOESY spectra to be used for

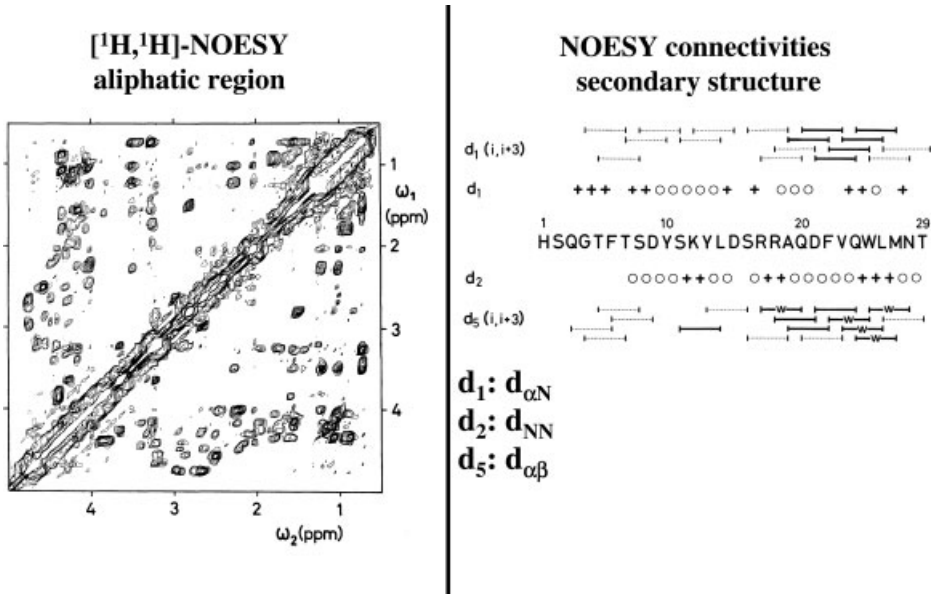

Figure 5. Sequential connectivities. Reprinted from *Journal of Molecular Biology*, **169**, Braun W, Wider G, Lee KH, Wüthrich K, Conformation of glucagon in a lipid–water interphase, pp 921–948, Copyright © 1983, with permission from Elsevier.

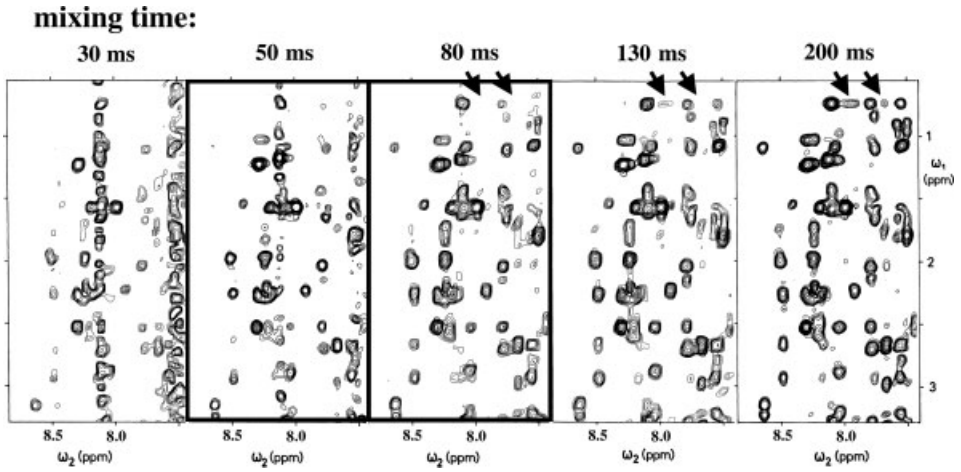

Figure 6. Spin-diffusion in NOESY spectra of micelle-bound glucagon.

the structure determination. For the proper selection of the mixing time we measured a series of NOESY spectra with different mixing times (Fig. 6). Based on the appearance of spin diffusion peaks in the 130 ms and the 200 ms spectra (arrows in Fig. 6) we used the 50 ms spectrum with some support from the 80 ms spectrum. Please note the t_1-noise in the 30 ms and partially in the 50 ms spectra, which shows the instabilities of the spectrometer in the old days and demonstrates how difficult it could be to find optimal and reproducible operating conditions.

The next problem to be solved was the conversion of resonance intensities in the NOESY spectra into distances. The information on the secondary structure obtained from the connectivity plot shown in Fig. 5 indicated that there is no globular rigid structure and that some flexible parts have to be expected. For this reason, a rigid model that correlates intensities and interproton distances r with a dependence of $1/r^6$ could not be used without further consideration. This rigid model we used only for NOEs between protons separated in the covalent structure by three or less torsion angles (Fig. 7). For all other NOEs the uniform averaging model was used.[16] This model takes into account the changing distance between two protons in non-rigid structures (Fig. 7). In this treatment, the distance between two hydrogen atoms is allowed to vary uniformly between a minimum distance r_m, and a maximum distance $r_m + R$. For a given nuclear Overhauser effect (NOE) the maximum distance obtained with the uniform averaging model is always longer than the corresponding distance obtained with the rigid model (Fig. 7). From the more rigidly structured helical part at the C-terminal it was clear that distances larger than 0.5 nm did not contribute NOEs. Based on this fact we used a maximal distance of 0.5 nm for the uniform averaging model. On the other hand, it could not be ruled out that the protons come very close and a minimal distance of 0.2 nm was chosen. Using nuclei with fixed distances (e.g. in methylene groups, aromatics) the models were calibrated and the NOE intensities were converted into distances according to the table in Fig. 7.

In 1981, computer power was rather limited, and for the structure calculation various measures had to be taken to reduce computing time. Inspection of the NMR data showed that no NOEs were observed between residues that would be further apart than five positions in the amino acid sequence. Therefore, to save computer time, the distance geometry calculations were performed separately for four overlapping segments of the polypeptide chain, i.e. 5–15, 10–20, 17–27 and 19–29. A further reduction of the computing time was obtained by the use of pseudo-atoms. A pseudo-atom could replace a methylene group, a methyl group or even a complete aromatic ring.[1,17] For each peptide segment a staggering number of 10 independent computer runs were made. The result of the computer runs that converged are shown in Fig. 8. This figure is also an example of the state of computer graphics in the early 1980s. Figure 8 shows for each segment a bundle for the backbone conformers and a bundle representing the backbone with sidechains. In addition, one conformer is plotted as a ball-and-stick model. The number of converged structures and the quality of the structure determination for the four segments varies, with root-mean-square deviation (RMSD) values between 0.85 and 2.1 (Fig. 9). Despite the complexity of the glucagon–DPC system, the result demonstrated for the first time that structure determinations of polypeptides and proteins by NMR were possible. A plot of the $\Phi-\Psi$ backbone torsion angles of representative structures confirmed that the structure calculation did produce sterically meaningful conformers (Fig. 9). The data points for glucagon in DPC micelles fall within or near the allowed areas. An encouraging result, since effective energy minimization was hampered by limited computing power. Not quite unexpectedly, glucagon in micelles was shown to be partially flexible and extended. The first determination of a globular structure of a polypeptide was to become the protein BUSI,[18] from which the NMR

NOESY		d (Å)† (rigid model)	d_{max} (Å)† (uniform averaging model)
τ_m (ms)	Intensity‡		
50	≥10	≤2·4	≤3·0
50	6–9	≤2·7	≤3·0
50	2–5	≤3·1	≤4·0
80	≥2	≤4·0	≤5·0

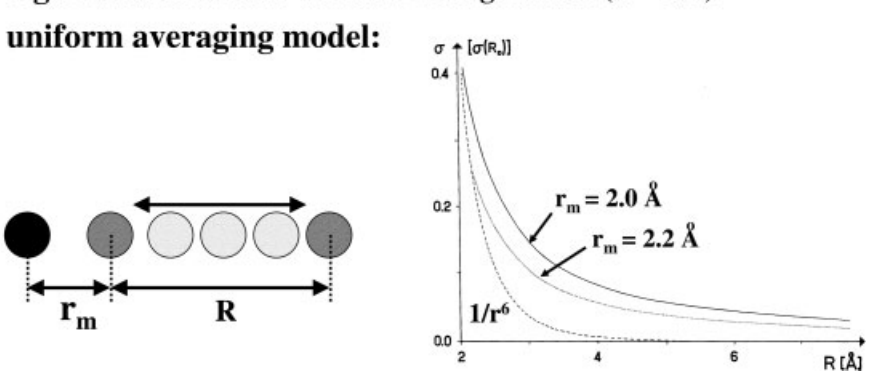

Figure 7. Conversion of NOE intensities into distances.

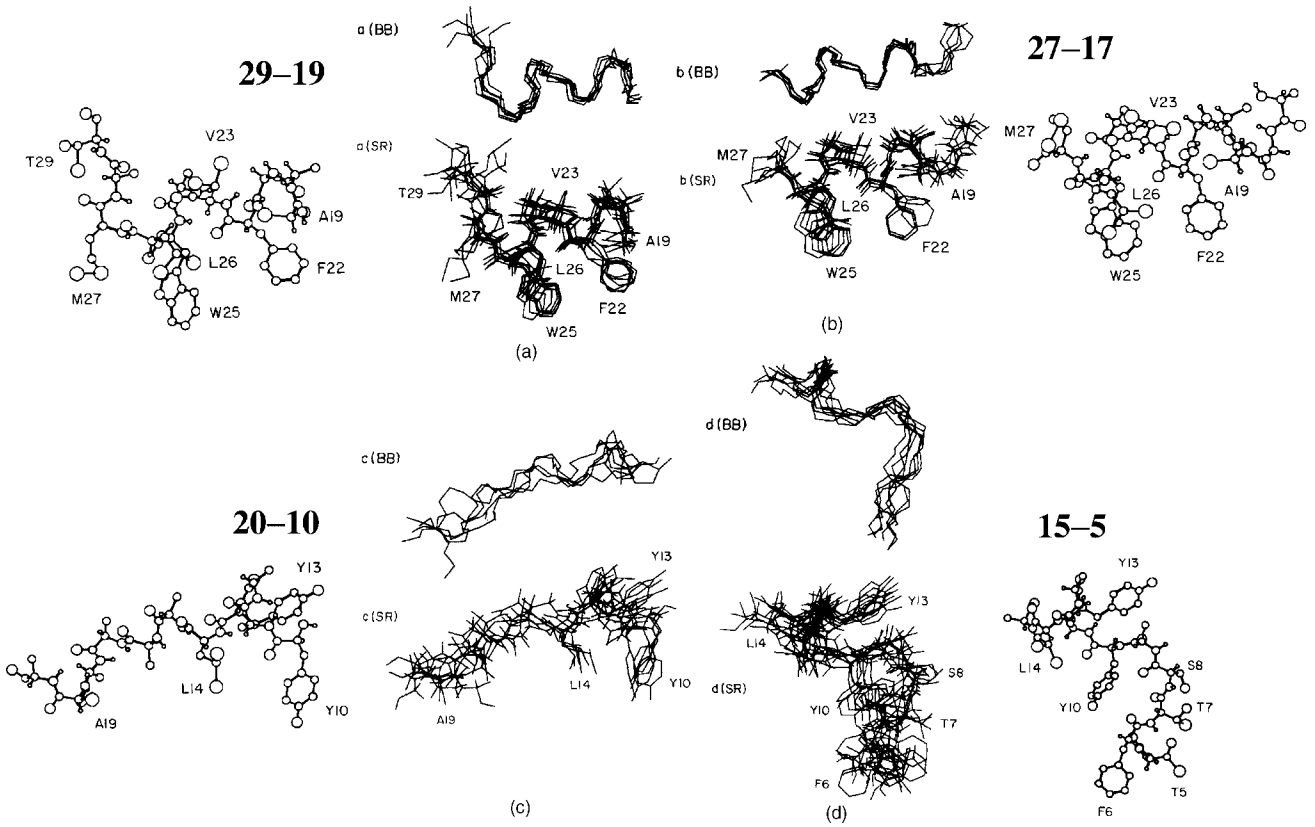

Figure 8. Glucagon structures. Reprinted from *Journal of Molecular Biology*, **169**, Braun W, Wider G, Lee KH, Wüthrich K, Conformation of glucagon in a lipid–water interphase, pp 921–948, Copyright © 1983, with permission from Elsevier.

Sterically acceptable conformers out of 10 calculations

MB-glucagon segment	Number of conformers	rmsd BB‡
5–15	8	1·52
10–20	5	2·08
17–27	5	0·85
19–29	6	1·15

BB: N, C^{α}, C'

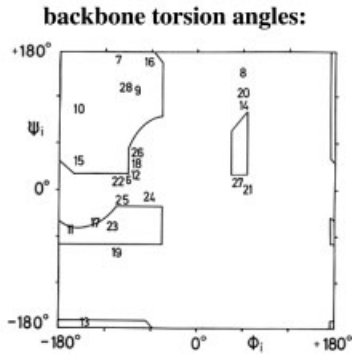

Figure 9. Characterization of the structures of micelle-bound glucagon. Reprinted from *Journal of Molecular Biology*, **169**, Braun W, Wider G, Lee KH, Wüthrich K, Conformation of glucagon in a lipid–water interphase, pp 921–948, Copyright © 1983, with permission from Elsevier.

resonance assignments[19] were determined in parallel to the structural work on glucagon bound to micelles.[17]

DISCUSSION

Including the studies of glucagon bound to DPC micelles, three structural studies of glucagon in different environments were available in 1981. The X-ray structure of glucagon trimers showed an α-helical conformation and in aqueous solution monomeric glucagon was shown to have a predominantly extended and flexible conformation. Figure 10 on the left presents a comparison of the polypeptide backbone structures of micelle-bound glucagon with the X-ray structure of glucagon.[10] The black circles in glucagon–DPC and the black triangles in the crystal structure mark the C^{α} positions indicated by the numbers. Obviously, the two

structures are markedly different except in the C-terminal part. For the NMR spectroscopists, this first structural study was already a confirmation that protein structures in solution and in single crystals could show distinctive differences. In our great enthusiasm we combined the four partial structures to a hypothetical complete structure of glucagon in DPC micelles (Fig. 10 on the right). One representation shows the backbone only, and the other includes sidechains as well; on the right-hand side the whole molecule is turned by 90 °C about a horizontal axis. The overall shape of the molecule is not reliably characterized by the NOE data. However, the backbone outlines approximately the curvature of the DPC micelles and, since glucagon in DPC micelles was found to be located near the surface,[20] the overall shape

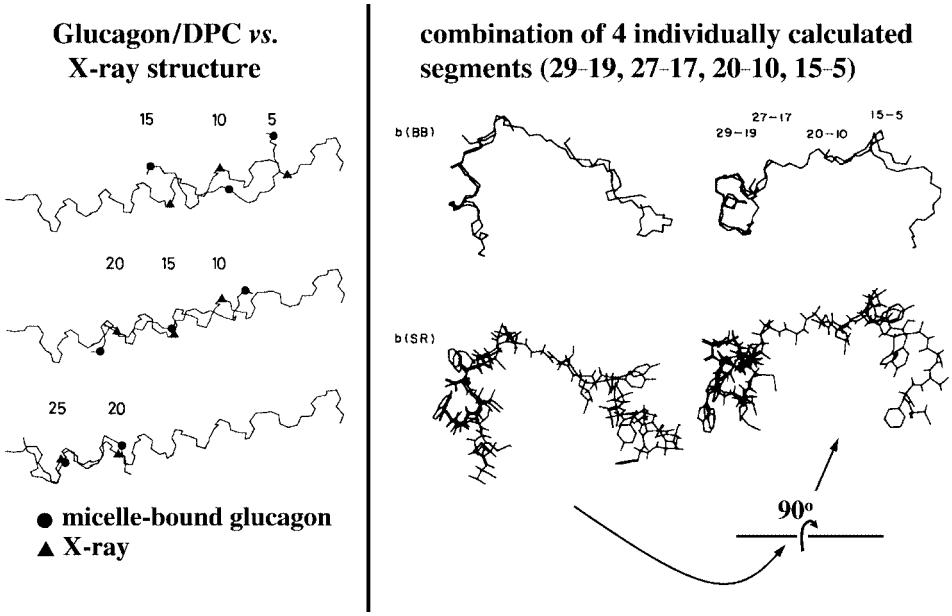

Figure 10. Description of the structures of micelle-bound glucagon. Reprinted from *Journal of Molecular Biology*, **169**, Braun W, Wider G, Lee KH, Wüthrich K, Conformation of glucagon in a lipid–water interphase, pp 921–948, Copyright © 1983, with permission from Elsevier.

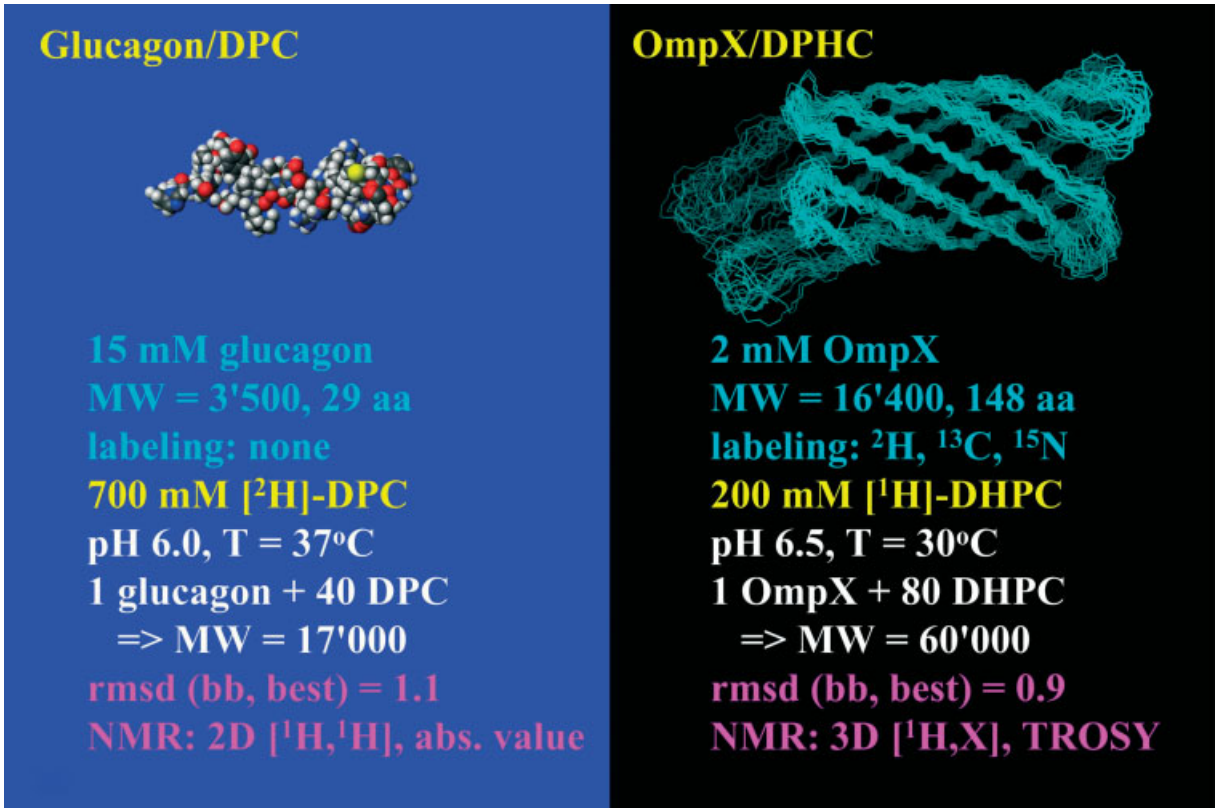

Figure 11. Working with mixed micelles.

Figure 12. Acknowledgment.

might nonetheless coincide with the micelle-bound polypeptide.

All the work described up to now was done more than 20 years ago, when I was a PhD student in the research group of Kurt Wüthrich. It may be interesting to establish ties to present-day work. Currently, we are working on another protein solubilized in micelles: the membrane protein OmpX reconstituted in dihexanoylphosphatidylcholine (DHPC) micelles with a total molecular weight of about 60 kDa.[21,22] Figure 11 presents a comparison of the two systems. The structure of glucagon–DPC was determined based on homonuclear 2D ^{1}H absolute value spectra with simple structure calculation programs. The structure of OmpX–DHPC was calculated with data obtained from phase-sensitive 3D heteronuclear spectra using sophisticated structure calculation programs. Surprisingly the RMSD of the best defined backbone segment is quite similar in both structures. This fact is also a consequence of the shortcomings inherent in a comparison of structures using only their RMSDs. The comparison of the two systems clearly demonstrates the development of the NMR techniques within the last 20 years: today we work with almost 10 times less concentrated samples on five times larger systems. This progress required substantial developments in NMR spectrometer hardware, computer hardware and software, biochemical methods and NMR techniques.[23–25] These ongoing developments make NMR still a very exciting, lively and very attractive field of research.

Acknowledgements

I thank Kurt Wüthrich for accepting me as a PhD student in 1978, for the opportunity to work at the forefront of 2D NMR during my PhD thesis and his continuous support. Without him I would not have spent half of my life working in the field of NMR at the ETH and in industry. In the glucagon project I worked together with Werner Braun, Kong Hung Lee and Adela Chreszczyk (Fig. 12) and in the OmpX–DHPC project together with Cesar Fernández and Christian Hilty; I thank all of them for their dedication. Special thanks go to Kuniaki Nagayama for teaching me the basics of 2D NMR and I thank him and Richard Ernst for the inspiring and enjoyable discussions on 2D NMR. Kurt Wüthrich initiated and guided all the projects described, and I want to use this opportunity to thank Kurt Wüthrich very much for many years of great collaboration.

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