Chimia 54 (2000) 627–632

ISSN 0009–4293

Using Structural Information of Peptides, Derived from NMR Spectroscopy, in Pharmaceutical Chemistry

Reto Bader, Mirjam Lerch, Gerd Folkers, and Oliver Zerbe*

Abstract: The significance of information gained from the solution structure of peptides for pharmaceutical research is demonstrated with two examples: the neuropeptide Y (NPY) hormone system and a undecapeptide designed for use as a chemical sensor. In the case of NPY, the structure of the homodimer and the mode of membrane association was determined. Thereby, it was discovered that the membrane/NPY interface is formed by the same hydrophobic residues that constitute the homodimer interface. Furthermore, in the membrane-bound state, the C-terminal helix is stabilized, which is of special functional importance for the C-terminal tetrapeptide. Receptor-subtype specificity of NPY mutants may be explained through pre-orientation of residues relative to the different membrane compartments. In the case of the undecapeptide designed for use in a chemical sensor, structural information from NMR helped us to design and optimize a peptide whose unligated form is unstructured in solution but adopts a unique helical fold upon addition of sulfate. The sulfate binding pocket is formed by the N-terminal first turn.

Keywords: Chemical sensors · G-protein coupled receptors · Neuropeptide Y · NMR · Pharmaceutical chemistry

Introduction

Nuclear magnetic resonance (NMR) has been extensively used in the past as an analytical tool in chemistry and pharmaceutical sciences to provide information on both the covalent and the three-dimensional structure of molecules. Similar to single-crystal X-ray diffraction, it delivers structural data at atomic resolution. Of particular importance for applications in life science subjects, however, is that it may be applied to molecules in solution under conditions that closely resemble those of physiological relevance. Following the pioneering work of Wüthrich and his coworkers in the 80s [1] the potential of the technique to resolve structures of peptides and proteins in solution has become increasingly apparent and a large number of coordinates that have been deposited in the Brookhaven data bank (pdb) [2] were derived by NMR. Peptides or proteins are frequent subjects of interest in pharmaceutical sciences and hence knowledge about their shape or about the exact location of residues that form contacts to drugs is highly desired. Unfortunately, biological NMR is presently limited to systems smaller than 30–60 kDa although substantial progress to tackle larger molecules has been made recently [3]. NMR is particularly attractive in fields that contain smaller but structured peptides or the class of membrane-interacting peptides/proteins, both of which are known to successfully resist trials for crystallization. To illustrate its use in pharmaceutical chemistry, two projects from completely different fields of pharmaceutical chemistry that both required the use of peptide NMR will be introduced.

*Correspondence: Dr. O. Zerbe
Institute of Pharmaceutical Sciences
Department of Applied Biosciences
ETH Zürich
Winterthurerstrasse 190
CH-8057 Zürich
Tel.: +41 1 635 60 81
Fax: +41 1 635 68 84
E-Mail: oliver.zerbe@pharma.ethz.ch

Deciphering the Role of Hormone-Membrane Interactions

About 70% of the pharmaceutically relevant drugs bind to G-protein coupled receptors [4]. Despite its biological importance, little structural information is available for these systems so far. We are presently investigating the neuropeptide Y (NPY)/Y_n-receptor (n = 1..6) system. NPY is a 36-amino acid residue, C-terminally amidated neurohormone [5] involved in the regulation of blood pressure [6] and food uptake [7]. NPY in solution is characterized by an α-helix comprising the second half of the molecule connected to a largely unstructured N-terminus [8][9]. So far, six different receptors have been discovered, five of which have been sequenced up to now [10]. They all belong to the class of G-protein-coupled receptors that are structurally characterized by seven *trans*-membrane helices interconnected to each other *via* loops on both membrane surfaces. Receptor-subtype specific ligands are highly desirable in

order to determine the exact pharmacological profile of each of them. Several receptor-specific NPY analogs have been synthesized so far and a large amount of mutational data are available from Ala scans of NPY [11]. We have recently characterized the solution structure and the pharmacological profile of [^{31}Ala, ^{32}Aib]-NPY that selectively binds to the Y_5-receptor with high affinity (IC_{50} 6 nM) [12]. The overall topology largely follows the fold of NPY. However, the ^{31}Ile→Ala and ^{32}Thr→Aib double mutation introduces flexibility into the backbone such that the C-terminal helix does not extend to the last residue Tyr36 as described in the solution structure of Monks *et al.* [8] but rather ends with a 3_{10} helical turn comprising residues Ile28 to Ala31. The latter is clearly seen from the (i,i+3) hydrogen bonds formed between the carbonyl oxygen of residue Ile28 and the amide proton of residue Ala31 instead of the (i,i+4) hydrogen bond commonly observed in regular α-helices (Fig. 1).

In light of the structural data from the Y_5-selective agonist and the binding affinity data of other NPY mutants at the various receptor subtypes, we have decided to study native NPY both in solution and in its membrane-bound form. NPY has a self-association constant of 1.6 +/–0.6 μM and therefore exists predominantly as a dimer at the concentrations required for NMR. After release from storage into the synaptic cleft, concentrations of NPY are still high and hence the dimer is expected to predominate. Both Cowley *et al.* [9] and Monks *et al.* [8] have tried to determine the dimer interface from intermolecular NOEs. However, for the particular case of NPY, we have found it difficult to use this strategy, because we have not been able to unambiguously assign all resonances from the aliphatic side-chains that are probably involved in making intermolecular contacts. Spectral overlap in this region of the spectra additionally precluded proper integration of the signals. Instead, we have mixed ^{15}N uniformly isotopically enriched NPY with an excess of a NPY mutant at natural (0.3%) ^{15}N abundance, in which Gln34 was replaced by the amino acid 4-amino-2,2,6,6-tetramethyl piperidine-1-oxyl-4-carboxylic acid (TOAC). This amino acid contains a (nitroxyl) spin label and spatial proximity of the spin label to other protons leads to highly efficient electron-spin relaxation. Signals from protons no more than about 10 Å apart are therefore highly broadened. Observation of reduction of signal intensities in [^{15}N,^{1}H]-HSQC spectra reveals positions of amide protons inside the other monomer subunit that are close to Gln34 in the dimer. Since the TOAC spin-label and ^{15}N nuclei are placed in different molecules, interpretation of the data is straightforward even in the presence of a monomer-dimer equilibrium. The most dramatic effects were seen on signals from residues Ala14, Ile28 and Thr32, which are inconsistent with any of the so far proposed dimer models. Instead we propose two different arrangements of NPY in the dimer: a parallel and an anti-parallel alignment of the helices, which form a dynamic equilibrium *via* a partially unfolded intermediate.

From Ala scans of NPY at the Y receptors it was obvious that the C-terminal residues ^{36}Tyr, ^{33}Arg and ^{35}Arg are essential and may not be substituted without significant losses in activity [10][11]. In addition, at the Y1 and Y5 receptors, some of the N-terminal residues are important as well. These residues probably form direct contacts with the receptors. The complexity of the mutational data is tremendous but some may be more easily explained when taking hormone-membrane interactions into account. Schwyzer has developed the Membrane Compartment Theory [13][14]. The theory states that membrane binding serves to direct residues into the correct compartments, *e.g.* the membrane, water interface or the hydrophobic interior, thereby pre-positioning them for receptor binding and possibly inducing the bio-active conformation. Furthermore, the receptor search is limited to lateral diffusion on the membrane surface. We have therefore decided to study the structure and dynamics of NPY on (perdeuterated) dodecylphosphocholine (DPC) micelles [15]. DPC has been especially developed for use in NMR [16] and allows the study of membrane–peptide interactions without broadening resonances to an extent that excludes high-resolution NMR experiments. The structure of NPY on DPC micelles is highly similar to the structure of (unligated) NPY in the solution dimer. However, significant differences do exist for the C-terminal tetrapeptide (see Fig. 2). In the membrane-bound form, this part of the hormone is structurally better defined. Although the data of Monks *et al.* [8] indicate that the α-helix extends up to residue Tyr36, our relaxation analysis shows that the C-terminal tetrapeptide is highly flexible. When comparing the solution data of Monks with our data from the membrane-bound form, Tyr36 is differently oriented such that it is close to the membrane-water interface in the micelle-bound form. In contrast, these residues would be directed into the aqueous compartment when the solution structure is docked onto the membrane.

To further characterize the NPY/DPC system we have probed the flexibility of the C-terminal helix using ^{15}N relaxation.

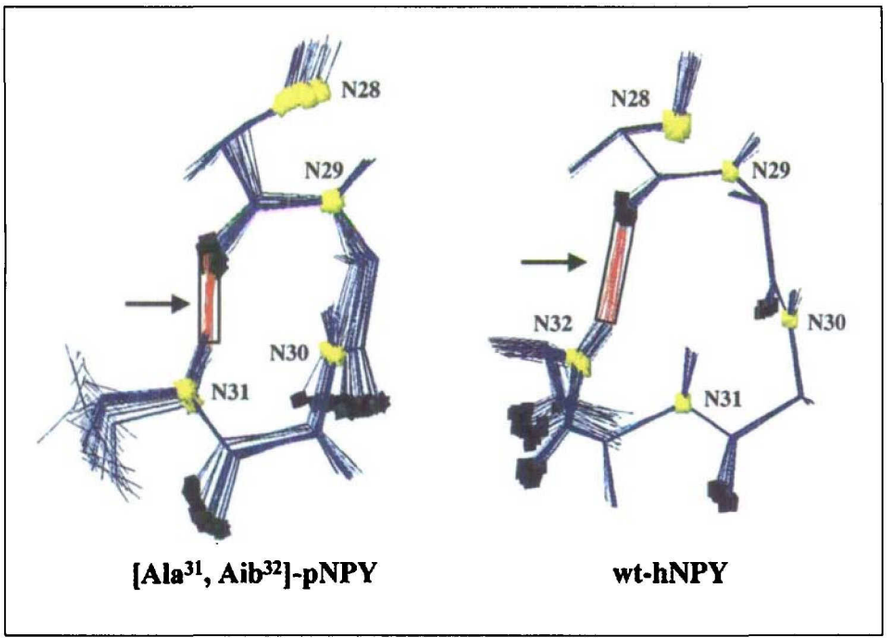

Fig. 1. Hydrogen bond pattern over the residues 28–32 of [Ala^{31}, Aib^{32}]-pNPY and hNPY. On the left, the 30 lowest energy solution structures are superimposed over the backbone heavy atoms of the residues 28–31. On the right, the structure of hNPY according to the data of Monks *et al.* [8] is depicted. The hydrogen bonds are boxed and their position is indicated by arrows.

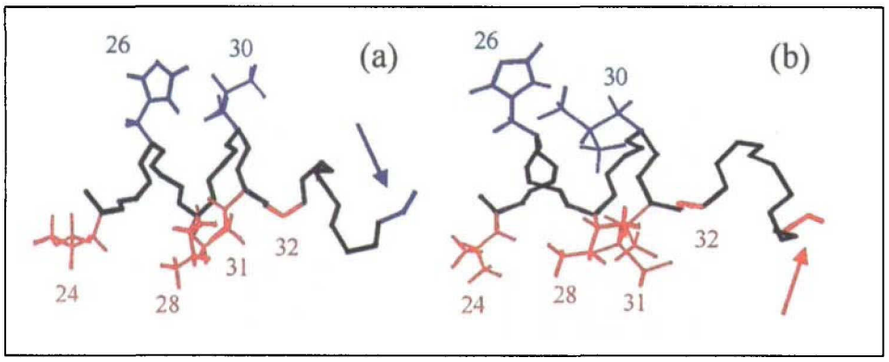

Fig. 2. One representative energy-minimized solution structure displaying residues Leu24-Tyr36 of NPY (a) in aqueous solution as determined by Monks *et al.* [8] and (b) bound to DPC micelles, using the backbone atoms (C^{α}, N and C') and side-chain atoms (with RMSD < 1.0 Å) as indicated. The position of backbone atoms of residue Tyr36 is indicated by an arrow.

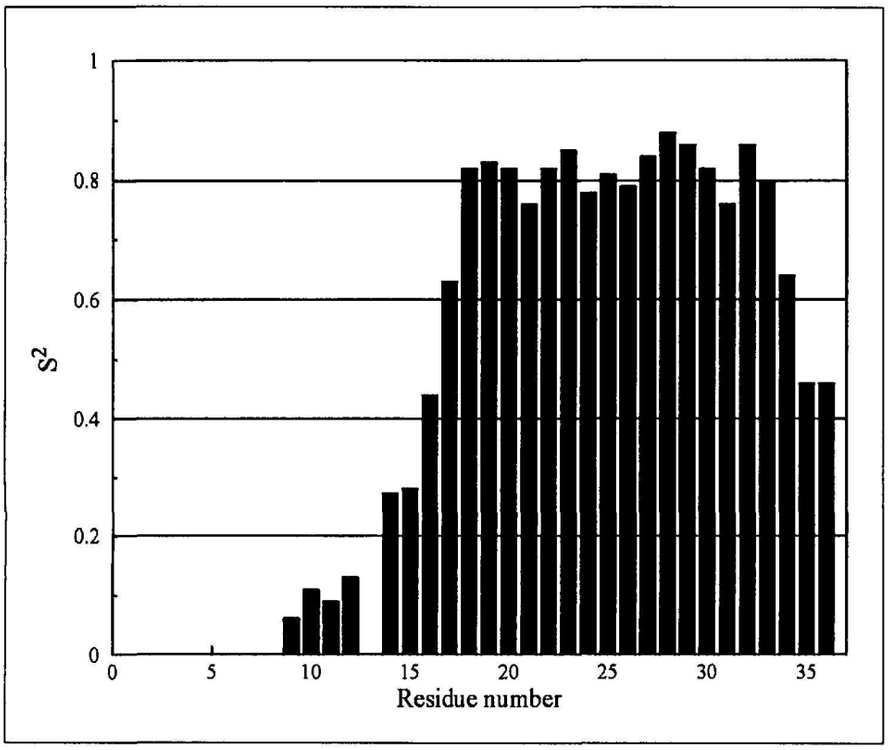

Fig. 3. Modelfree order parameters S^2 for pNPY in DPC solution from ^{15}N spin relaxation measurements as a function of the protein sequence.

The latter supplies information on the magnitude of motions of $^{1}H,^{15}N$ bond vectors in a coordinate system that is synchronized to overall tumbling. The information is similar to that derived from the B-factors known in X-ray crystallography but additionally provides data on the time-scale of these motions. From these measurements we could demonstrate that the α-helix is significantly stabilized in the membrane-bound form and that the C-terminal tetrapeptide in particular becomes more structured upon binding to the micelle. We have interpreted the relaxation data in terms of the modelfree formalism developed by Lipari and Szabo [17][18]. Fig. 3 displays the order parameters of NH backbone vectors along the peptide sequence and yields typical values of stably folded helical proteins for residues 18–33, and somewhat lower values for the C-terminal tripeptide. In fact, the values for the latter are still high enough to show that the helix is present even at the C-terminus for most of the time.

In that context it is also important to have information about the exact orientation of the molecule with respect to the membrane surface. We have again utilized spin labels for this purpose. 5- and 12-doxylstearates, which intercalate into the micelle hydrophobic interior, are commercially available. In case of the former, the spin label is located close to the charged DPC head-groups. We have again computed relative peak volumes derived from $[^{15}N,^{1}H]$-HSQC spectra recorded in the presence and in the absence of the spin-label 5-doxylstearate (Fig. 4). The data revealed a 3–4 periodicity on the signal intensities indicating that the C-terminal helix is placed parallel to the membrane surface. Interestingly, this effect can be largely removed upon addition of $CaCl_2$ at physiological concentrations of NaCl, which reduces the affinity of the hormone to the membrane. Most prominent signal reductions were observed for residues Leu17, Tyr21, Leu24, Arg25, Ile28, Asn29 and Tyr36. This indicates that binding of NPY to the membrane proceeds *via* hydrophobic contacts originating from intercalation of the long, hydrophobic side-chains of Leu, Ile and possibly the terminal Tyr-amide residues into the hydrophobic interior. These residues anchor the hormone onto the membrane surface and orient the residues Arg33 and Arg35, which are believed to make direct contacts with the receptor, into the aqueous compartment. We therefore believe that binding to the membrane serves two purposes: Firstly, it helps to direct the hormones to the receptor by restricting the receptor search to lateral diffusion along the membrane surface. Secondly, it pre-orients residues into the correct compartments and thereby facilitates receptor binding. Deconvolution of the relative importance of residues for membrane anchoring and for forming receptor contacts is highly desired and would allow to design NPY mutants with even better subtype-specificity on a more rational basis.

Designing Sulfate-Binding Peptides

Designing ionophores based on small organic molecules has proven to be difficult and requires substantial efforts in synthetic organic chemistry. Moreover, its practical use is still hampered by a manifold of limitations such as inadequate sensitivity, selectivity or lifetime [19–21]. In contrast, a variety of enzymes may be found in nature that selectively bind oxoanions such as sulfate, phos-

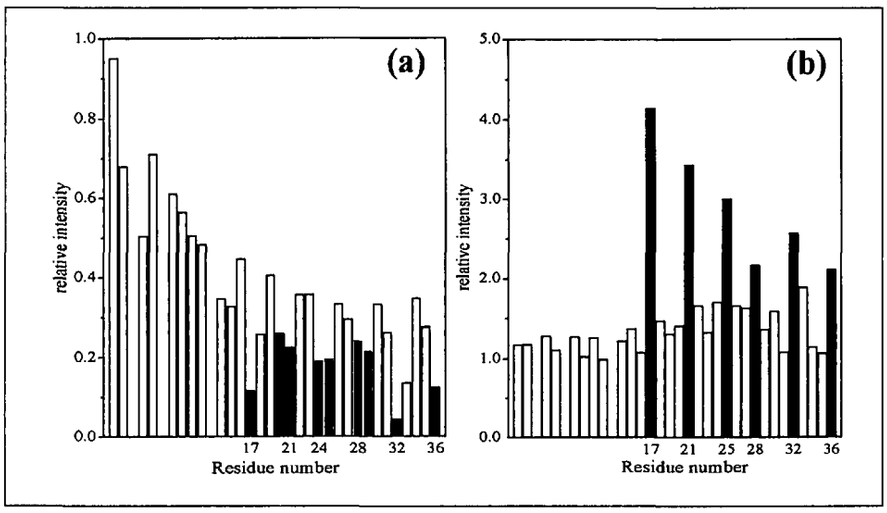

Fig. 4. Relative signal intensities in [^{15}N,^{1}H]-HSQC spectra of NPY in DPC solution (a) in the presence of the spin-labeled 5-doxylstearic acid with respect to a reference spectrum without spin-labeled stearic acid, and (b) in the presence of spin-labeled 5-doxylstearic acid plus 5 mM $CaCl_2$ and 150 mM NaCl with respect to a reference spectrum containing only the spin-labeled stearic acid and 150 mM NaCl.

phate or nitrate. Unfortunately, these enzymes have limited stability and are difficult to immobilize for use in a chemical sensor. We have therefore tried to develop peptides that are constructed based on the naturally occurring ion-binding epitopes and to subsequently modify them for practical application in a chemical sensor.

The design of the peptides was derived from the phosphate-binding protein Purin Nucleoside Phosphorylase (PNP), an enzyme that is involved in the reversible phosphorolysis of nucleotides [22][23]. The residues Ser33, Arg84, His86 and Ser220, which are hydrogen-bonded to the ligand [24], form the anion-binding site in the enzyme. These residues were flexibly linked through Gly and Leu residues and a number of undecapeptides were synthesized by solid-phase synthesis. A first screening was performed by CD spectroscopy and revealed the undecapeptide **1** (^{1}Ser^{2}Gly ^{3}Gly^{4}Leu^{5}Arg^{6}Leu^{7}His^{8}Leu^{9}Gly^{10}Leu^{11}Ser) as a successful first candidate [25]. This peptide is unstructured in solution in the absence of sulfate but becomes helically folded for residues 3–7 upon addition of one equivalent of sulfate. The structure calculation using restraints derived from a 400 ms NOESY at 280 K and $^{3}J_{HN\alpha}$ scalar coupling constants converged to an average RMSD of 0.97 Å and 2.1 Å for the backbone and side-chain heavy atoms of residues 3–8, respectively. The helical folding is conveniently recognized from inspection of the H^N,H^N spectral region of the NOESY (Fig. 5), which is devoid of clear crosspeaks in case of the unligated peptide but displays contacts of sequential amide protons typically encountered in helices for the sulfate-bound form. Further evidence stems from reduced $^{3}J_{HN\alpha}$ scalar coupling constants

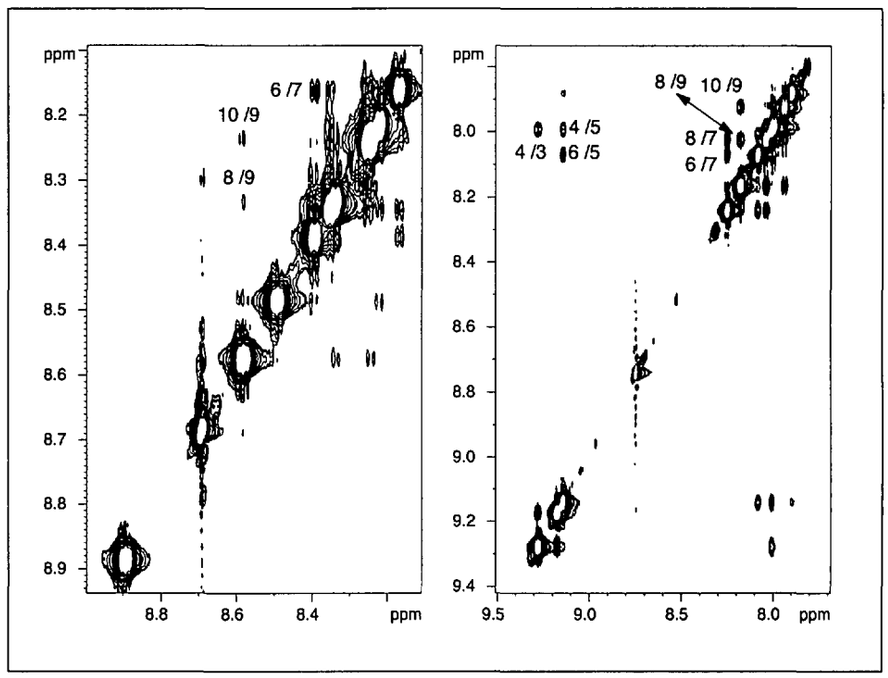

Fig. 5. Expansions of 400 ms NOESY spectra of free (left) and SO_4^{2-}-complexed (right) peptide **1**. Annotation of crosspeaks refers to the sequence positions of the amide protons.

and differences of the chemical shifts of H^{α} and H^N protons in the presence and in the absence of sulfate (Fig. 6). The latter additionally provides information about which protons are likely to be coordinated to the sulfate ion.

To investigate the specificity of sulfate binding, we have titrated **1** with aliquots of sodium sulfate solution and monitored the spectral changes. Interestingly, signals from N-terminal residues are exchange-broadened up to about 0.8 equiv. whereas those from the C-terminal residues remain essentially unchanged. After further addition of the anion, signals sharpen again and adding more than about 1 equiv. causes very little alterations in the spectra. Furthermore, the chemical shifts of the carbon-bound imidazole ring protons from His7 undergo very little change so that participation of His7 in sulfate binding is unlikely. We were also not able to detect structural changes upon lowering the pH to about 4, and insolubility of the peptide at basic pH precluded its characterization at pH 8.5.

Since proton NMR spectroscopy cannot deliver data about the exact binding mode of the sulfate we have docked the sulfate into the solution structure using the recently described Lamarckian genetic algorithm (GA) starting from randomly positioned sulfate [26]. This automated flexible docking procedure suggests a preferred clustering of the anion around the N-terminal half of the peptide (Fig. 7).

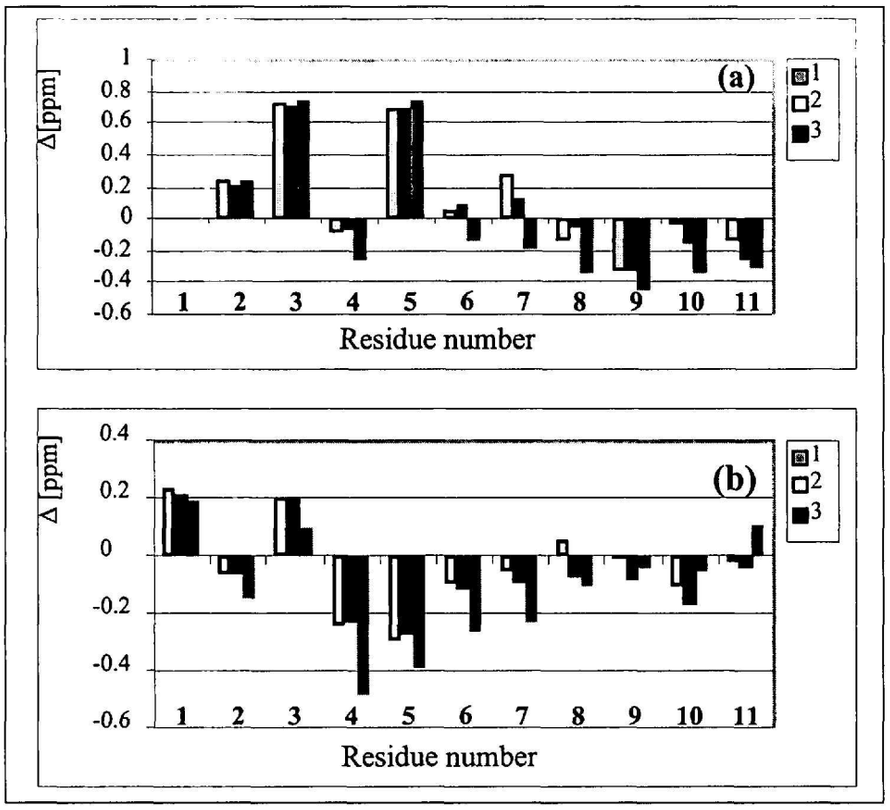

Fig. 6. Chemical shift differences of signals ($\Delta\delta = \delta_{PS} - \delta_P$) in ppm *vs.* the sequence. Top: H^N shifts. Bottom: H^α shifts.

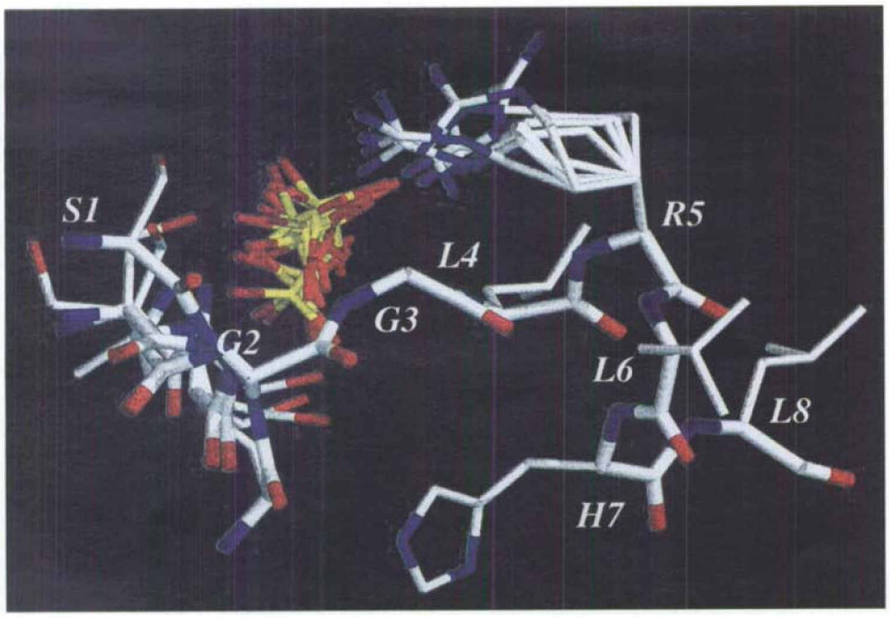

Fig. 7. Ensemble of docked sulfate complexes superimposed over the backbone atoms 3–5 and displaying the N-terminal part (residues 1–5) of the peptide-sulfate complex.

In particular, salt bridges to the Ser1 N-terminal amide group and to Arg5 side-chain atoms, as well as hydrogen bonds to Gly2, Gly3, (Leu4) nitrogen atoms and/or Ser1, Arg5 side-chain atoms are common to the ten best orientations found.

We have further tried to characterize the stability of the sulfate-peptide complex using a 0.8 ns MD simulation with explicit (methanol) solvent. A peptide-sulfate complex from the ensemble of docked structures was extracted and placed into a methanol box and forced to charge neutrality by adding a sodium ion. After the usual equilibration procedure of the peptide complex in the methanol box, 0.8 ns of unrestrained molecular dynamics was computed with the AMBER 6 forcefield at 280 K [27]. We have monitored the hydrogen bonds formed between peptide atoms and sulfate and the hydrogen bonds within the peptide in order to determine the mode of sulfate binding or the stability of the helix, respectively. In both cases hydrogen bonds were broken transiently but quickly reformed. The sulfate ion rotated about one of its symmetry axis but was coordinated by the same protons from the peptide throughout the simulation, except that a hydrogen bond between the γ-H of Ser1 and an oxygen from sulfate is formed only part of the time. Neither loss of sulfate nor any major unfolding of the helix was observed during the time-course of the MD simulation.

We have synthesized further derivatives of the peptide **1** to gain insight into the importance of residues for sulfate binding. To improve the solubility of the peptide we have replaced Ser11 by Lys. Two peptides were synthesized (**2**, ^{1}Ser^{2}Gly^{3}Gly^{4}Leu^{5}Arg^{6}Trp^{7}His^{8}Leu^{9}Gly ^{10}Trp^{11}Lys and **3**, ^{1}Ser^{2}Gly^{3}Gly^{4}Leu^{5}Arg ^{6}Trp^{7}His^{8}Leu^{9}Trp^{10}Leu^{11}Lys) that displayed better solubility in methanol and whose backbone fold was almost identical to **1**. The corresponding proton spectra in the presence and absence of sulfate were very similar amongst the series of peptides (Fig. 8) indicating that the sulfate complexes are structurally very similar and that coordination proceeds via the same atoms. However, solubility of **2** and **3** was sufficient to detect weak $d_{\alpha\beta}(i,i+3)$ and $d_{\alpha N}(i,i+4)$ NOEs that are diagnostic for a helical backbone fold [1]. Moreover, we have prepared a series of derivatives of **1** in which residues important for forming hydrogen bonds to sulfate were replaced by alanine. Characterization of these peptides is presently in progress in our laboratory to better understand the importance of the individual residues. From the data that we have recorded on these mutants it is clear that the residues Ser1 to Arg5 are important and that residues in the C-terminal part of the peptide are allowed to be altered without loss of the sulfate-binding capability. This finding is of particular importance since the system may then be modified, *e.g.* by coupling it to a polymeric support or by incorporating other features that will allow the use of optical spectroscopy to detect the structuring of the peptide.

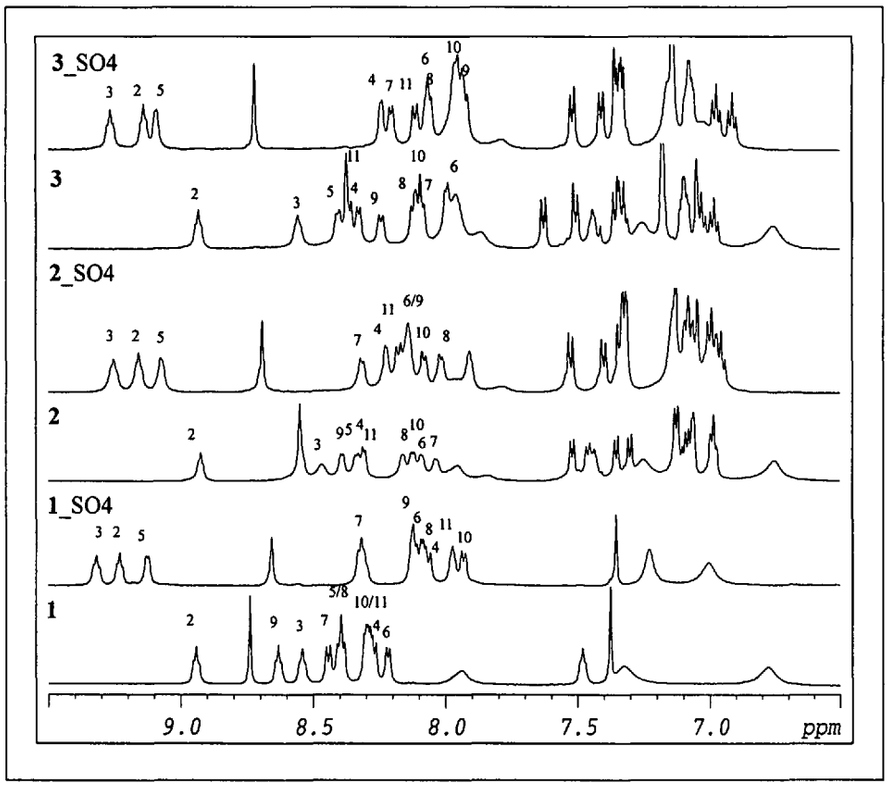

Fig. 8. 1D spectra displaying the region of the amide and aromatic protons of peptides **1** to **3** in the presence or absence of sulfate (see labels at upper left corner). Numbering of the signals refers to the sequence positions of the respective amide proton.

Initiation is an important step for forming helices. N-capping residues forming side-chain hydrogen bonds to the backbone atoms of the first turn significantly help to stabilize the first helix winding [28–30]. We suspect that the sulfate ion is taking over the stabilizing role of these N-capping residues by forming appropriate hydrogen bonds to backbone amide protons of residues 2–4. Backbone carbonyl groups are thus optimally oriented for inducing the first helical turn.

Conclusions

We have introduced two projects that have largely profited from the structural data provided by peptide NMR. In both cases the investigated systems were limited in size and hence data evaluation was achieved in a reasonably short time. Furthermore, the structural information that was derived from the NMR experiments was very fruitful to explain structural features that may be connected to receptor subtype specificity in the case of the NPY/Y-receptor system or that guided us to optimize the sequence of the sulfate-binding peptides in a rational way. The interplay between peptide synthesis/molecular biology required to produce the peptides and the structure elucidation by NMR provides a efficient route to further modify the systems. Of course, other applications of NMR in pharmaceutical chemistry such as the SAR by NMR technology recently introduced by Fesik [31] or the structure elucidation of (larger) proteins of pharmaceutical interest are also successfully and frequently exploited at the moment.

Received: September 6, 2000

[1] K. Wüthrich, 'NMR of Proteins and Nucleic Acids', Wiley, New York, **1986**.
[2] F.C. Bernstein, T.F. Koetzle, G.J.B. Williams, E.F. Meyer, Jr., M.D. Brice, J.M. Rodgers, O. Kennard, T.M.T. Shimanouchi, *J. Mol. Biol.* **1977**, *112*, 535–542.
[3] N. Tjandra, A. Bax, *Science* **1997**, *278*, 1111–1114; K. Pervushin, R. Riek, G. Wider, K. Wüthrich, *Proc. Nat. Acad. Sci USA* **1997**, *94*, 12366-71.
[4] A.G. Beck-Sickinger, *Drug Discovery Today* **1996**, *1*, 502–513.
[5] D. Larhammar, *Regul. Pept.* **1996**, *62*, 1–11.
[6] L. Grundemar, R. Hakanson, *Gen. Pharmacol.* **1993**, *24*, 785–796.
[7] B.G. Stanley, S.F. Leibowitz, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3940–3943.
[8] S.A. Monks, G. Karagianis, G.J. Howlett, R.S. Norton, *J. Biomol. NMR* **1996**, *8*, 379–390.
[9] D.J. Cowley, J.M. Hoflack, J.T. Pelton, V. Saudek, *Eur. J. Biochem.* **1992**, *205*, 1099–1106.
[10] M.C. Michel, A.G. Beck-Sickinger, H. Cox, H.N. Doods, H. Herzog, D. Larhammar, R. Quirion, T. Schwartz, T. Westfall, *Pharmacol. Rev.* **1998**, *50*, 143–150.
[11] A.G. Beck-Sickinger, H.A. Wieland, H. Wittneben, K.D. Willim, K. Rudolf, G. Jung, *Eur. J. Biochem.* **1994**, *225*, 947–958.
[12] C. Cabrele, M. Langer, R. Bader, H.A. Wieland, H.N. Doods, O. Zerbe, A.G. Beck-Sickinger, *J. Biol. Chem.*, in press.
[13] R. Schwyzer, *Biopolymers* **1995**, *37*, 5–16.
[14] R. Schwyzer, *J. Mol. Recognit.* **1995**, *8*, 3–8.
[15] R. Bader, A. Bettio, A.G. Beck-Sickinger, O. Zerbe, *J. Mol. Biol.*, in press.
[16] J. Lauterwein, C. Bösch, L.R. Brown, K. Wüthrich, *Biochim. Biophys. Acta* **1979**, *556*, 244–264.
[17] G. Lipari, A. Szabo, *J. Am. Chem. Soc.* **1982**, *104*, 4546–4559.
[18] G. Lipari, A. Szabo, *J. Am. Chem. Soc.* **1982**, *104*, 4559–4570.
[19] A. Bianchi, E. Bowman-James, K. García-España, 'Supramolecular chemistry of anions', Wiley, New York, **1998**.
[20] P. Chakrabati, *J. Mol. Biol.* **1993**, *234*, 463–482.
[21] U.E. Spichiger, 'Chemical sensors and biosensors for medical and biological applications', Wiley-VCH, Weinheim, **1998**.
[22] M. Friedkin, H.M. Kalckar, in P.D. Boyer, H. Lardy, K. Myrback (Eds.), 'The Enzymes', Vol. V, Academic Press, New York **1961**, 237–255.
[23] T.A. Krenitsky, *Mol. Pharmacol.* **1967**, *3*, 526–536.
[24] S.E. Ealick, S.A. Rule, D.C. Carter, T.J. Greenhough, Y.S. Babu, W.J. Cook, J. Habash, J.R. Helliwell, J.D. Stoeckler, R.E. Parks, S. Chen, C.E. Bugg, *J. Biol. Chem.* **1990**, *265*, 1812–1820.
[25] C. Demuth, O. Zerbe, D. Rognan, R. Söll, A.G. Beck-Sickinger, G. Folkers, U.E. Spichiger, manuscript in preparation.
[26] G.M. Morris, D.S. Goodsell, R. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639–1662.
[27] W.D. Cornell, P. Cieplak, C.I. Bayly, I.R. Gould, K.M. Merz, D.M. Ferguson, D.C. Spellmeyer, T. Fox, J.W. Caldwell, P.A. Kollman, *J. Am. Chem. Soc.* **1995**, *117*, 5179–5197.
[28] S. Penel, E. Hughes, A.J. Doig, *J. Mol. Biol.* **1999**, *287*, 127–143.
[29] L.G. Presta, G.D. Rose, *Science* **1988**, *240*, 1632–1641.
[30] J.S. Richardson, D.C. Richardson, *Science* **1988**, *240*, 1648–1652.
[31] S.B. Shuker, P.J. Hajduk, R.P. Meadows, S.W. Fesik, *Science* **1996**, *274*, 1531–1534.