Volume 278, number 2, 160–166 FEBS 09353 January 1991

ISSN 0014-5793/91/000588

Solution structure of μ-conotoxin GIIIA analysed by 2D-NMR and distance geometry calculations

Karl-Heinz Ott[1], Stefan Becker[2], Robert D. Gordon[2] and Heinz Ruterjans[1]

[1]*Institut für Biophysikalische Chemie, J.W. Goethe Universität Frankfurt am Main, Germany* and [2]*Max-Planck-Institut für Biophysik, Abteilung Molekulare Membranbiologie, Frankfurt am Main, Germany*

Received 29 August 1990, revised version received 11 October 1990

We have investigated the structure of μ-conotoxin GIIIA by 2D NMR methods. The assignment of ^{1}H NMR spectra and a quantitative analysis of NOE and J-coupling data are presented. These results were used for the calculation of secondary structure elements of μ-conotoxin GIIIA. Distance geometry calculations were carried out to define the global folding of the peptide.

μ-Conotoxin GIIIA, Geographutoxin, NMR, 2D, Distance geometry, Peptide synthesis, *Conus geographus*

1 INTRODUCTION

μ-Conotoxin GIIIA (GIIIA), a peptidic neurotoxin from the marine snail *Conus geographus* selectively blocks Na channels of skeletal muscle, but not neuronal or heart sodium channels [1,2]. This peptide can be used as specific ligand to probe the biochemical mechanisms by which the toxin blocks the sodium conductance [3]. The three-dimensional structure of the peptide will allow an evaluation of the structure-function relationship of the toxin with the channel protein.

Conotoxin GIIIA consists of 22 amino acids with 3 disulfide bonds (Fig. 1). The synthesis and characterization of GIIIA have been published [3–5] and more recently, Hidaka et al. [6] determined the disulfide linkages for GIIIA. To determine the three-dimensional solution structure, high resolution 2D ^{1}H NMR experiments were carried out. Structures which are consistent with experimental data were calculated with distance geometry algorithms, which can be used as start conformations for energy minimizations and molecular dynamics calculations [7].

Correspondence address H. Ruterjans, Institut für Biophysikalische Chemie, Theodor Stern Kai 7, Haus 74A, D-6000 Frankfurt/70, Germany

Abbreviations Hyp, 4-trans-hydroxy-L-proline; NOE, nuclear Overhauser enhancement; dNA, distance between Hα(i) and HN(i); dAN, distance between Hα(i) and HN(i+1); dNB, dBN, dNN similar for Hβ and HN; NMR, nuclear magnetic resonance; DQF COSY, double quantum filtered correlated spectroscopy; NOESY, 2-dimensional (2D) nuclear-Overhauser-enhancement spectroscopy; ROESY, rotating frame Overhauser enhancement spectroscopy; E. COSY, exclusive COSY; TOCSY, total correlated spectroscopy; HPLC, high pressure liquid chromatography; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulphonyl.

2 MATERIALS AND METHODS

Samples were synthesized by two different strategies. In the first strategy, the preparation, purification and characterization of GIIIA is described in [4], while the synthesis using the second strategy is described in [5]. The HPLC purified product was lyophilized several times from (0.1 % v/v) TFA/water to remove ammonium acetate. The samples were prepared by dissolving 5 mg and 108 mg of the dried powder in 0.5 ml of H_2O/5% D_2O.

Phase sensitive 2D NMR spectra were recorded at 293K, 303K and 315K with a Bruker 500 MHz NMR spectrometer. NOESY [8], ROESY [9,10] and TOCSY [11] spectra were acquired with 512 increments in t_1 and 2K data points in t_2. Mixing times in NOESY and ROESY spectra range from 50 ms to 300 ms. TOCSY spectra were recorded with MLEV-17 spin lock field of 7.2 kHz strength [12] and mixing times between 12 and 80 ms. Transversal Overhauser experiments were obtained with a continuous 2.5 kHz B_1 field. DQF-COSY [13,14], E. COSY [15,16] and 1D NMR spectra can be used to determine values for dihedral angles. For correlated spectra with multi-quantum filtering, 1K points for f_1 and 4K data points for f_2 were acquired. The spectral width was 5000 Hz for all spectra. The data matrices were zero filled to give final matrices of 2K real points in both dimensions. Interatomic distances were deduced from a series of 3 NOESY spectra with mixing times of 50, 125 and 200 ms.

After processing to a final 2K × 2K matrix a contour plot was used to define boxes around each cross-peak. The cross-peak volumes as a function of the mixing time were automatically fitted to a single-exponential function [17]. From the initial build-up rates, the inter-proton distances could be calculated [18]. The distances were scaled by using a mean value of the rates of cross-peaks of geminal protons. The error on these distances is smaller than 0.15 Å.

To determine the conformation of the individual amino acid, NOEs between amide, Hα and Hβ proton resonances and amide protons of the following residue (d_{NN}, $d_{\alpha N}$, $d_{\beta N}$) as well as intraresidual ones ($d_{N\alpha}$, $d_{N\beta}$) were used [19,20] in combination with coupling constants (^3J) [21,22] (Fig. 3). The Karplus equation with parameters given in [21,23] was used to calculate dihedral angles from coupling constants. Applying a combination of these methods, possible ranges for the ψ, φ and χ^1 angles can be obtained.

The DISMAN program [24] was used to calculate structures from amino acid sequence and experimental constraints. The concept of pseudoatoms [25] was used to define distances to protons in methylene and methyl groups, which are not explicitly assigned.

Published by Elsevier Science Publishers B.V. (Biomedical Division)

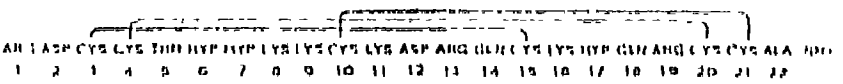

Fig. 1. Primary sequence of α-conotoxin GIIIA indicating the disulfide bridges.

3. RESULTS

By reverse phase HPLC, mass spectrometric and competition binding experiments the sample synthesized by the published methods was proven to be pure with high activity (data not shown). The NMR spectra of the first sample indicated more than one conformation of this species (Fig. 2). This was indicated by the occurrence of more than the expected cross-peaks in the fingerprint region. As an example, two different sets of resonance positions could be identified for the protons of Thr^5 and Ala^{21}. The overlapping resonances at 8.3 ppm and 4.3 ppm indicated a random coil structure for at least parts of the molecule. In the 1D ^1H NMR spectrum additional resonances for aromatic protons were found. We have assumed that they are residual Mtr protecting groups of arginine side chains. The improved synthesis led to a complete cleavage of protecting groups and to a simplified purification and preparation. In the spectra of this sample there is no evidence for more than one stable conformer.

TOCSY experiments yield a map of through-bond couplings for the individual amino acids. The first step in resonance assignment was the identification of the spin systems. NOESY and ROESY experiments contained information about interproton distances. Sequential assignment of the amino acid resonances was performed by identifying connectivities between amide proton resonances of one amino acid and Hα and Hβ proton resonances of adjacent ones in NOESY and ROESY spectra. This assignment was confirmed by sequential amide-amide proton resonance connectivities. For Hyp the Hδ protons were used instead of amide protons in other amino acids. Resonances overlapping with the water proton resonance at a given temperature were assigned in the spectra recorded at a different temperature. The integrated NOESY cross-peaks were examined for possible overlapping of the resonances and for unusual line shapes. 80 interproton distances could be clearly assigned, about 40 NOEs had to be discarded for the first analysis. Unfortunately, most of the long range NOEs important for determining the global folding were ambiguous due to resonance overlap. For all pairs of proton resonances all NOESY and ROESY spectra were searched for corresponding cross-peaks. In cases where unambiguously no NOEs were observed, the lower limit of this distance was set to 3.5 Å. The resulting more than 700 'non-NOEs' were used as upper limit constraints in DISMAN calculations. A qualitative comparison of corresponding NOE and ROE intensities indicated that the contribution of differences in the correlation time is small. Coupling constants were extracted from 1D, double quantum filtered COSY and E. COSY experiments (Table I).

At first, it was only possible to assume a disulfide bridge between residues 3 and 15, which was confirmed by a NOE between Cys^1 Hα and Cys^{15} Hβ, and between Cys^{15} Hβ and Cys^1 Hβ.

NOE connectivity patterns for regular secondary structural elements like α-helices or β-pleated sheets [26] were not found. The stereospecific assignment of the Hβ protons was based on modelling and calculations of substructures using information on short range NOEs and $^3J_{\alpha\beta}$ coupling constants, also with respect to the interpretation of ^3J couplings the determination of a preliminary substructure was necessary. Since there is no singularity in the Karplus equation only a combination of distances and coupling constants can restrict the different possibilities of dihedral angle ranges. This will be demonstrated in the following example.

The NOE between Cys^1 Hα and Cys^1 HN was weak which indicated a negative φ value. $^3J_{NH}$ of Cys^1 is small, limiting the φ value to a range of $-60°$ to $0°$. A small $^3J_{\alpha\beta}$ and a large $^3J_{\alpha\beta''}$ is observed in *gauche*^{2}-*trans*^{1} or *trans*^{2}-*gauche*^{1} conformations. We were able to distinguish between these two by comparing NOEs between the Hβ protons and the amide proton of the following residue. For the *gauche*^{2}-*trans*^{1} conformation two strong NOEs should be observed, while for the other conformation one strong and one weak NOE would be expected. In GIIIA a strong NOE of one of the Cys^1 Hβs to the HN of Cys^1 restricted the ψ value of Cys^1 to a range of $+40°$ to $-140°$. Since the distance between Cys^3 Hα and Cys^4 HN is larger than about 3 Å a further restriction to the range of $-40°$ to $+40°$ for the ψ angle was possible.

In the same way the conformations of the other Cys residues were analyzed. The two possible assignments for the Hβ protons of Cys^4 led to χ^1 angles of $-60°$ or $0°$. This range could be used as a dihedral restraint. For Cys^{10} we assume a χ^1 angle of $-60°$. Also for Cys^{20} the stereospecific assignment of Hβ resonances was possible and a χ^1 value of $+60°$ was determined.

The two adjacent Hyp residues were expected to have a distinct secondary structure. A very strong cross-peak between the Hβ of Thr^5 and one of the Hδ of Hyp^6 indicated a *trans* peptide bond. It was not possible to find any NOE between protons of Hyp^6 and Hyp^7, which showed that the planes of the two Hyp rings are arranged at an angle of almost 90° with respect to each other and the peptide bond is in the *cis* configuration. The NOEs expected between the two hydroxyprolines in this

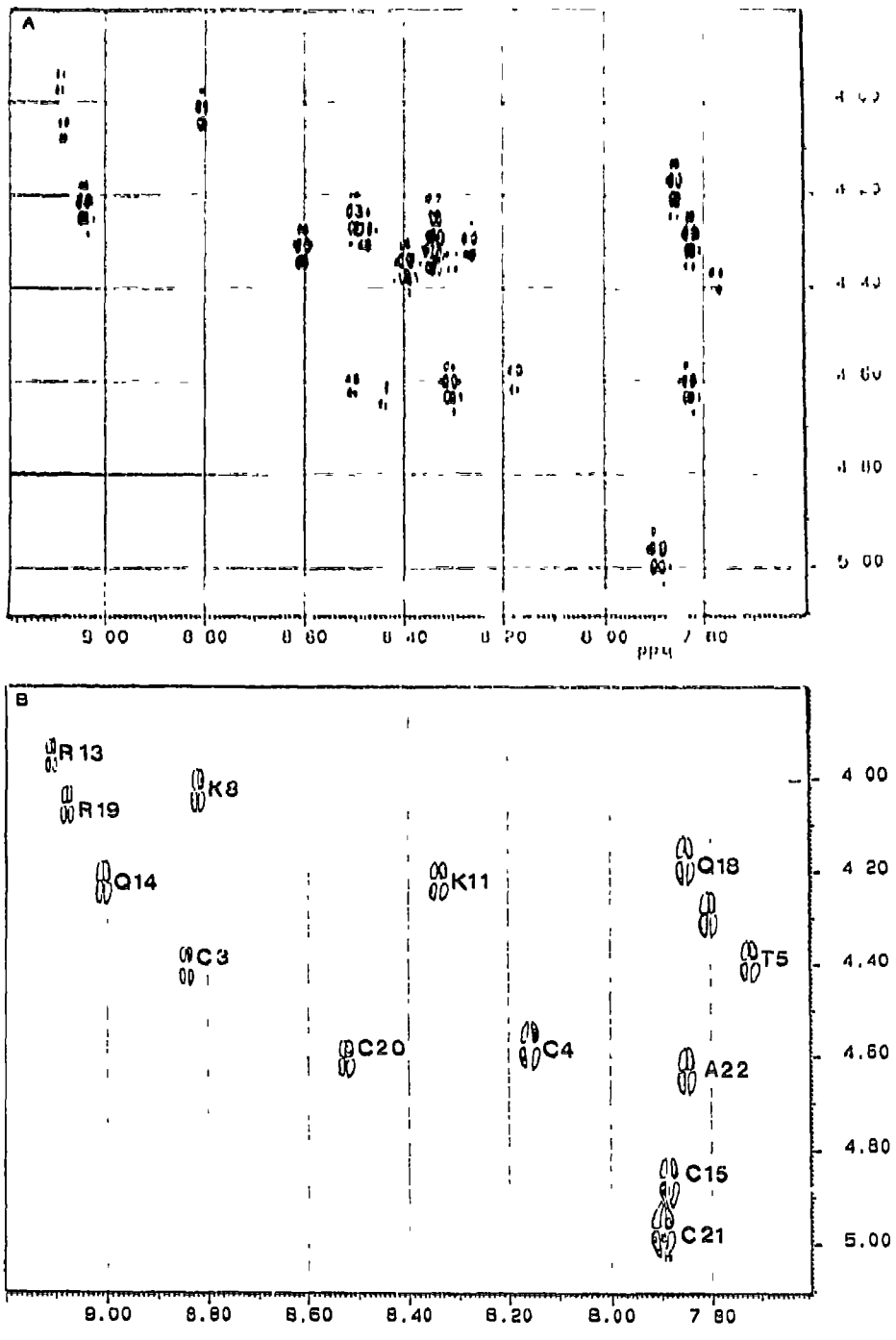

Fig. 2. HN-Hα fingerprint region of the COSY spectra of (A) GIIIA synthesized by the method described in [4], and (B) after modifying the synthesis [5]. In (B) the assignment of the resonances is indicated.

conformation were not detected because of overlap of both the Hα protons and the Hβ2 resonances. Lys[16] and Hyp[17] are connected by a *trans* peptide bond which was indicated by NOEs between Lys[16] HN and both Hyp[17] Hδ protons.

Additional HN-Hα NOEs between amino acid residues separated by two sequential steps confirmed the modelling of the loop structures. These NOEs were observed between residues 3 and 5, 10 and 12, 13 and 16, 17 and 19, 18 and 20. With this additional information, the calculations resulted in loop structures in between Asp[2] and Tyr[5]. This loop resembles a type I β-turn. Turn structures were also found for the sequence Cys[10] to Arg[14]. Two kinks in the course of the backbone

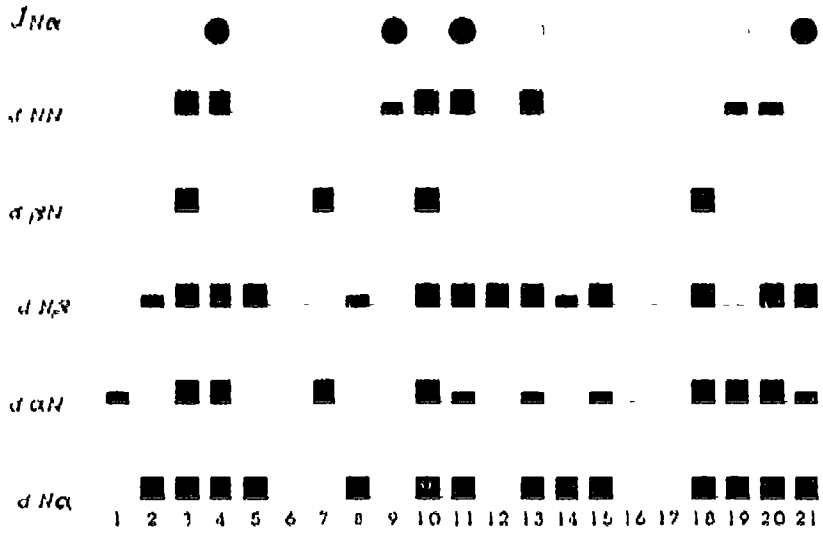

Fig. 1. Sequential connectivities and ^3J coupling constants for GIIIA. Small and large bars indicate weak and strong NOEs, respectively. Filled circles symbolize J coupling values larger than 7.5 Hz, open circles indicate couplings smaller than 6.5 Hz.

between Hyp[17] to Cys[21] were determined (Fig. 4). Having defined these substructures, the identity of some long range NOEs, which were ambiguous due to resonance overlap could be confirmed: Asp[2] Hβ and Cys[21] Hβ are overlapping. In the modelled loop no NOE is expected between Asp[2] Hβ and Cys[3] Hα, therefore confirming the assignment of this NOE to Cys[21] Hβ. This is in agreement with the disulfide bonds between Cys[4] and Cys[21].

Finally, starting from initial random structures 30 different conformations were obtained in a distance geometry calculation. 80 upper limits, 780 lower limits, 10 dihedral ranges and 18 distances for the disulfide bridges restricted the possible conformations (Fig. 5).

Table 1

Chemical shifts and coupling constants for GIIIA at 293K

		HN	HA	HB2 HB3	HG	others	$^3J_{N\alpha}$	$^3J_{\alpha\beta}$	$^3J_{\alpha\beta'}$
1	Arg	7.93	4.03	(1.90,1.98)	1.52,1.45				
2	Asp	9.06	4.75	(3.06,3.00)			6.7		
3	Cys	8.97	4.3	2.97 2.62			5.4	2-3[b]	10-11[a]
4	Cys	8.12	4.52	(3.50,2.83)			7.8	<12[b]	3-4[a]
5	Thr	7.63	4.37	4.93	1.22			< 5	
6	Hpr	-	4.8	1.97 2.37	4.63	4.03,3.79			
7	Hpr	-	4.72	2.28 2.38	4.57	3.67,3.48			
8	Lys	8.87	3.98	(1.93,1.82)	1.25	1.63,2.98	5.0		
9	Lys	8.2					8.2		
10	Cys	7.36	4.3	3.18 2.77			< 5	12[a]	5[b]
11	Lys	8.38	4.16	(1.93,1.72)	1.3,1.4	1.6,2.93		8.2	
12	Asp	7.96	4.68	3.06 2.96			7.5	~ 5	~ 5
13	Arg	9.23	3.88	(1.95,1.83)	1.67,1.63			< 5	
14	Gln	8.98	4.16	(2.42,2.03)			5.5		
15	Cys	7.90	4.8	(3.05,3.15)			<5		
16	Lys	7.65	4.03	(1.92,1.82)	1.4,1.5	1.6,2.9		7.5	
17	Hpr	-	4.6	2.36 1.9	4.44	3.21,3.785	6-8	6-8	
18	Gln	7.88	4.12	(2.47,2.32)	2.0		6.0		
19	Arg	9.14	4.01	(1.95,1.83)	1.70		<5		
20	Cys	8.55	4.55	3.82 3.03			5.5	4[b]	4[b]
21	Cys	7.88	4.92	(3.19,2.93)			9.2		
22	Ala	7.92	4.25	1.40				7.5	

Chemical shifts are reported in ppm relative to external TSP. Coupling constants are given in Hz. Values for β proton resonances given in brackets were not selectively assigned.

[a] Values obtained directly from COSY or 1D spectra.

[b] Coupling constants determined from the E. COSY spectra.

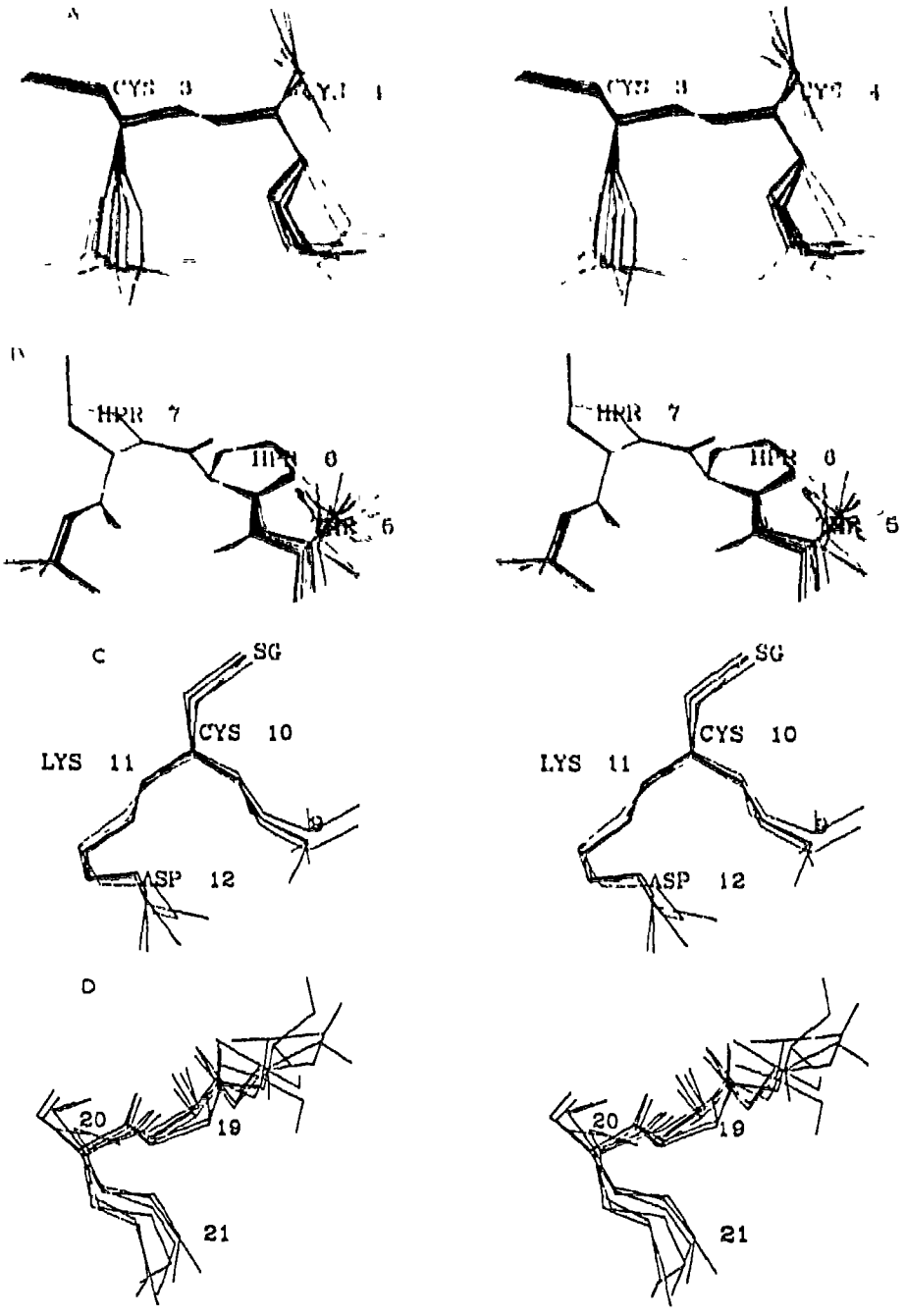

Fig 4 Stereo views of the superimposed substructures calculated using the DISMAN program The substructures are taken from the overall conformations of GIIIA, they are superimposed at the backbone atoms of the displayed fragment (A) Sequence Asp^{2} to Tyr^{5}, (B) sequence Tyr^{5} to Lys^{8}, (C) sequence Lys^{9} to Asp^{12}, (D) sequence Gln^{18} to Cys^{21}

4. DISCUSSION

In order to elucidate the three-dimensional solution structure of GIIIA, and here we present details of the secondary structure elements and a hypothesis for its global folding

First the NMR spectroscopy was used as an analytical tool to optimize the GIIIA synthesis in order to obtain a pure sample, which was the basis for sequential assignment and accurate structural analysis. The high dispersion of the amide proton resonances, many $^{3}J_{NA}$ coupling constants different from 7.5 Hz and sometimes large differences in the chemical shift of the Hβ' and Hβ'' proton resonances indicated a defined folded structure. The secondary structure elements are restricted to different loop structures. The loops between Asp^{2} and Tyr^{5}, Cys^{10} and Arg^{13}, and Hyp^{17} and Cys^{20} can be

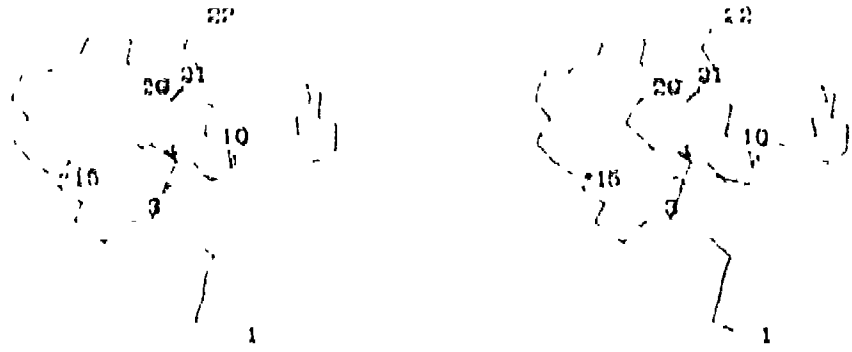

Fig. 5. This stereo view depicts the backbone atoms of the GIIIA conformation which best fits the experimental constraints.

determined with very high accuracy due to a high amount of experimental data. Differences between distances derived from NOE values and from the distance geometry calculations of these peptide segments were found to be smaller than 0.3 Å. This is well within the range of experimental error which arises from the signal-to-noise ratio and the approximations made in calculating distances from NOEs. It should be emphasized that the use of 'non-NOEs' mainly reduces the number of wrong conformations in the calculations. A structure prediction for two of these three loops using the Chou and Fasman rules [27] has been published [6], which we were able to confirm experimentally.

For determining the complete tertiary structure the detailed structural analysis of parts of the molecule it was necessary to include dihedral restraints derived from J-couplings [21,23]. Minor residual violations of the experimental constraints were found in all calculated conformations. The structure with the lowest error value is presented in Fig. 5. As outlined above, there are certainly differences in the accuracy of determining the conformation of peptidic segments. Of course, the more distance and dihedral angle constraints can be obtained, the more the conformation can be precisely determined. For the more flexible parts of molecules fewer distances and dihedrals are found to occur. In GIIIA accurate fitting of the experimental results were obtained for those parts of the molecule, where a lot of constraints were defined. Structural flexibility in parts of the molecule will result in experimental values representing an average of fast exchanging conformers. Some of the experimental results cannot be interpreted with a rigid structure model. Some observations confirm this difficulty in the interpretation. The intensity of the NOESY and ROESY signals is relatively low and the signals are broad. We have some indication, that near Lys^9 more than one conformer is present. A line broadening of the Lys^9 side chain proton resonances indicates a slow exchange between these conformations.

A more detailed analysis of the structural and dynamic properties will be presented in a forthcoming paper.

Acknowledgements: We thank Prof. H. Michel for his generous advice and assistance. We thank Dr. W. Braun for a version of the DISMAN program. This work was supported by the Deutsche Forschungsgemeinschaft Grant SFB 169, Projects C4 and B6, and by a Leibnitz Program grant to Prof. H. Michel.

REFERENCES

[1] Gray, W. R., Olivera, B. M. and Cruz, L. J. (1988) Annu. Rev. Biochem. 57, 665–700.
[2] Becker, S. and Gordon, R. D. (1990) in: (Herken, H. and Hucho, F., eds) Selective Neurotoxicity, Handbook of Experimental Pharmacology, Springer-Verlag, Berlin (in press).
[3] Cruz, L. J., Kupryszewski, G., LeCheminant, G. W., Gray, W. R., Olivera, B. M. and Rivier, J. (1989) Biochemistry 28, 3437–3442.
[4] Becker, S., Atherton, J. and Gordon, R. D. (1989) Eur. J. Biochem. 185, 79–84.
[5] Becker, S., Liebe, R. and Gordon, R. D. FEBS Lett. (in press).
[6] Hidaka, Y., Sato, K., Nakamura, H., Kobayashi, J. and Shimonishi, Y. (1990) FEBS Lett. 264, 1, 29–32.
[7] Scheek, R. M., Gunsteren, W. F. van and Kaptein, R. (1989) in: Methods in Enzymology, vol. 177, pp. 204–218, Academic Press, New York.
[8] Macura, S. and Ernst, R. R. (1980) Mol. Phys. 41, 95–117.
[9] Bothner-By, A., Stephens, R. L., Lee, J., Warren, C. D. and Jeanloz, R. W. (1984) J. Am. Chem. Soc. 106, 811.
[10] Bax, A. and Davis, D. G. (1985) J. Magn. Res. 63, 207–212.
[11] Davis, D. G. and Bax, A. (1985) J. Am. Chem. Soc. 107, 2820–2821.
[12] Bax, A. and Davis, D. G. (1985) J. Magn. Res. 65, 355–360.
[13] Piatini, U., Sørensen, O. W. and Ernst, R. R. (1982) J. Am. Chem. Soc. 104, 6800.
[14] Shaka, A. J. and Freeman, R. (1983) J. Magn. Res. 51, 169.
[15] Griesinger, C., Sørensen, O. W. and Ernst, R. R. (1985) J. Am. Chem. Soc. 107, 6395–6396.
[16] Griesinger, C., Sørensen, O. W. and Ernst, R. R. (1987) J. Magn. Res. 75, 474–492.
[17] Peppermans, H., Trouwé, D., Binst, van G., Boelens, R., Scheek, R. M., Gunsteren, van W. F. and Kaptein, R. (1988) Biopolymers 27, 323–338.
[18] Solomon, I. (1955) Physical R. 99, 559–565.
[19] Billeter, M., Braun, W., Wuthrich, K. (1982) J. Mol. Biol. 155, 321–346.
[20] Sherman, S. A., Andrianov, A. M. and Akhrem, A. A. (1987) J. Biol. Str. Dyn. 4, 869–884.
[21] Hyberts, S. G., Marki, W. and Wagner, G. (1987) Eur. J. Biochem. 164, 625–635.
[22] Basus, V. J. (1989) Methods Enzymol. 177, 132–149.
[23] Pardi, A., Billeter, M. and Wüthrich, K. (1984) J. Mol. Biol. 180, 741–751.

[24] Braun, W. and Go, N. (1985) J. Mol. Phys. 186, 611–626.
[25] Wuthrich, K., Billeter, M. and Braun, W. (1984) J. Mol. Biol. 169, 949–961.
[26] Richards, F.S. (1991) Adv. Prot. Chem. 34, 167–339.
[27] Chou, P.Y. and Fasman, G.D. (1978) Adv. Enzymol. 47, 45–148.
[28] Kuprov, I.W. and James, T.L. (1984) J. Magn. Res. 57, 401–426.
[29] Kessler, H., Griesinger, C., Lautz, J., Muller, A., Gunsteren, van W.F. and Berendsen, H.J.C. (1988) J. Am. Chem. Soc. 110, 3393–3396.