Tetrahedron Vol. 48, No. 41, pp. 8855-8868, 1992
Printed in Great Britain

0040-4020/92 $5.00+.00

CONFORMATION OF MurNAc-L-Ala-D-iGln (MDP) AND OF A CONSTRAINED ANALOG USING ^{1}H NMR DATA AND MOLECULAR MODELING

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(*Received in USA* 24 *July* 1992)

Key words: Glycopeptide, NMR, three-dimensional structure, nuclear Overhauser effects, molecular modeling.

Abstract: *The structures of N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and of its analog, N-acetylmuramyl-L-alanyl-3-carbomethoxymethyl-D-proline methyl ester (MAP) in DMSO have been investigated by one- and two-dimensional ^{1}H NMR spectroscopy. Using NMR-derived dihedral angles and interproton distances as constraints, an extensive computer search (SYBYL) of the optimal structures of the two glycopeptides has been performed. Low energy conformations clustered into several groups for both MDP and MAP. The formation of a β-turn by hydrogen bonding of the carbonyl group of the N-acetyl and the amino group of the L-alanyl moiety was present in all conformations for both glycopeptides. The calculated lowest energy conformation of MDP does not show the presence of a second β-turn. An S-shaped structure similar to a β-turn was observed in MAP. The temperature and solvent dependences of the chemical shifts of the amide protons support the existence of a single β-turn and of an equilibrium between the different structural clusters.*

INTRODUCTION

N-Acetylmuramyl-L-alanyl-D-isoglutamine or MDP (Fig. 1) has been recognized as the shortest molecular structure responsible for expressing the activity of Freund's complete adjuvant.[1,2] Several NMR studies have been performed on MDP and MDP analogs to determine the ratio of the α and β anomers[3-6] and to characterize their conformations.[7-11] Elements of conformational structure were obtained from the temperature dependence of the amide chemical shifts, from three-bond coupling constants (dihedral angles) and from nuclear Overhauser effect measurements (interproton distances). On the basis of the temperature dependence of the amide chemical shifts in MDP, hydrogen bonding between the carbonyl group of the N-acetyl (NAc) of the muramyl moiety and the amine group of the L-alanyl (L-Ala) was proposed.[7,9] Hydrogen bonding involving the terminal amino group of D-isoglutamine (D-iGln) and the carbonyl group of D-Lac

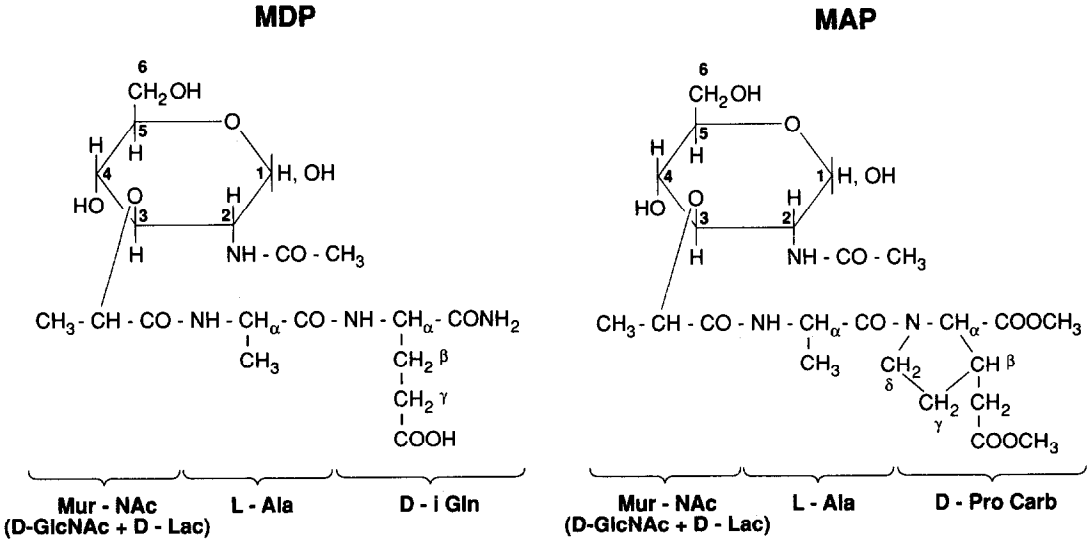


Fig. 1. Chemical structures of MDP and MAP.

leading to the formation of an S-shaped structure, has also been proposed but is not clearly supported by the NMR data (*vide infra*).[7] Coupling constant determinations allowed to calculate possible dihedral angles in the sugar ring and in the peptide chain.[7] Of the eight NOE connectivities reported, none involved long range distances.[8] Despite the lack of clear support from the temperature dependence of the chemical shift data for a second hydrogen bond, the solution conformation of the peptide portion of MDP has been proposed to consist of two type II β-turns forming an S-shaped structure.[7] The existence of two relatively weak hydrogen bonds was also postulated from the temperature dependence of the amide chemical shifts for non-glycosylated MDP analogs with the same constituent isomers i.e. D-lactoyl (D-Lac) and L-Ala.[6] In murabutide, where the terminal D-iGln of MDP is replaced by a D-glutamine-*n*-butyl ester, only the hydrogen bond involving the NAc and the L-Ala groups can be seen.[7] In longer chain MDP analogs, both hydrogen bonds may be lost in the formation of a larger cyclic structure.[11]

In order to obtain more detailed conformational information on the three-dimensional structure of the MDP molecule and of its synthetic analog N-acetylmuramyl-L-alanyl-3-carbomethoxymethyl-D-proline methyl ester (abbreviated as MAP, Fig. 1),[12] we utilized two-dimensional ^{1}H NMR and computer molecular modeling techniques. The rigid proline ring of this analog was expected to mimic the structure of the second β-turn proposed for MDP.[7] Structural data were derived from rotating frame Overhauser enhancement spectroscopy (ROESY) experiments, from vicinal coupling constants and from the temperature dependence of the chemical shifts of the amide protons. A conformational search was carried out to obtain three-

Table I. Chemical Shift Data for the Glycopeptides MDP and MAP in DMSO at 20°C

Moiety[a]	Proton	Chemical shift (ppm)			
		MDP		MAP	
		α-anomer	β-anomer	α-anomer	β-anomer
D-GlcNAc	H-1	4.96	4.42	4.99	4.45
	H-2	3.66	3.48	3.63	3.57
	H-3	3.46	3.30	3.43	3.25
	H-4	3.25	3.20	3.25	3.23
	H-5	3.62	3.11	3.61	3.14
	H-6	3.61	3.46	3.64	3.46
	H-6'	3.46	3.67	3.50	3.46
	OH-1	6.57	6.65	6.56	6.68
	OH-4			5.26	5.26
	OH-6	4.44	4.56	6.58	
	AcNH	8.05	7.95	8.08	7.97
	$AcCH_3$	1.79	1.79	1.80	1.80
D-Lac	Hα	4.28	4.17	4.34	4.22
	CH_3	1.23	1.24	1.24	1.24
L-Ala	NH	7.64	7.53	7.68	7.54
	Hα	4.28	4.25	4.62	4.62
	CH_3	1.22	1.24	1.18	1.18
D-iGln	NH	8.18	8.11		
	Hα	4.14	4.14		
	Hβ	1.94	1.94		
	Hβ'	1.70	1.70		
	Hγ,γ'	2.20	2.20		
	NH_2 H_E	7.30	7.30		
	NH_2 H_Z	7.02	7.07		
D-ProCarb	Hα			3.99	3.99
	Hβ			2.49	2.49
	Hγ			1.71	1.71
	Hγ'			2.12	2.12
	Hδ			3.59	3.59
	Hδ'			3.65	3.65
	CH_3OCO			2.50	2.50
	$CarbCH_2$			2.13	2.13
	$CarbCH_3$			2.50	2.50

[a] D-GlcNAc: N-acetylmuramyl; D-ProCarb: 3-carbomethoxymethyl-D-proline methyl ester.

dimensional structures compatible with the NMR data. Different clusters of low energy structures were obtained which suggest the existence of a S-shaped structure in MAP but not in MDP.

RESULTS

The assignments of all observable resonances of MDP and MAP are presented in Table I. Except for the hydroxyl protons, whose chemical shifts may be sensitive to the water content of the solvent, all MDP chemical shifts are in good agreement with the values previously reported in the same solvent.[7] In contrast to the broad signals observed for the HO-4 and HO-6 of the sugar moiety of MDP, these signals are sharp in the case of MAP. Chemical shift differences can be observed in all moieties of MDP between the α- and β-anomer but no difference can be observed in the proline moiety of MAP between the two anomers. It should also be noted that, when two inequivalent protons exist on a same carbon, they cannot be stereospecifically assigned.

The vicinal coupling constant values $^3J_{NH,H}$ for the NAc and L-Ala moieties of MDP and MAP are reported in Table II. Dihedral angle values were calculated using the relation developed by Bystrov *et al.*.[14] The two possible negative values for the NAc group are different between the α and β anomers for both MDP and MAP (Table II). Positive dihedral angle values are not given since they are associated with the higher energy structures for L-amino acids. For the L-Ala moiety, the same angles are calculated for the α and β anomers in both glycopeptides. The vicinal coupling constants could not be measured for the MDP D-iGln moiety due to the broadness of the peaks. The measurable coupling constant values $^3J_{NH,H}$ are in the range of 6.5-8.5 Hz and are therefore consistent with limited chain flexibility.

Table II. $^3J_{NH,H}$ and Possible Dihedral Angle Values Calculated for MDP and MAP[a]

Compound	Moiety	$^3J_{NH,H}$ (Hz)		Dihedral angles (°)	
		α-anomer	β-anomer	α-anomer	β-anomer
MDP	NAc	6.57	7.88	-161 -79	-153 -87
	L-Ala	6.99	6.92	-159 -81	-159 -81
MAP	NAc	7.69	8.42	-155 -86	-150 -90
	L-Ala	8.36	8.42	-150 -90	-150 -90

[a] Dihedral angles were calculated using the relation of Bystrov *et al.*[14] Positive values have been omitted because they are associated with the higher energy left handed helix conformations.

Temperature-dependence coefficients of the chemical shifts of the amide protons are presented in Table III. For the α-anomers, the temperature coefficients of the L-Ala amide protons of MDP and MAP are

Table III. Temperature-Dependence Coefficients of the Chemical Shifts of the Amide Protons of MDP and MAP

Compound	Moiety	Temperature coefficients (x 10^{-3} ppm/K)[a]	
		α-anomer	β-anomer
MDP	NAc	-4.80	-2.00
	L-Ala	-2.65	-0.61
	D-iGln NH	-4.10	
	D-iGln NH_2 H_Z	-4.61	-4.94
	D-iGln NH_2 H_E	-3.90	-4.00
MAP	NAc	-6.19	-2.30
	L-Ala	-3.19	-0.94

[a] Measured between 300 K and 350 K.

compatible with medium strength hydrogen bonding. Except in the case of the NAc group of MAP, all the other amide temperature coefficients are suggestive of weak hydrogen bonding (intramolecular or with DMSO), their values ranging between -3.90 and -4.80 x 10^{-3} ppm/K. The NAc group of MAP, however, does not appear to be involved in hydrogen bonding. For the β-anomers, very low temperature coefficient values were measured for the amide protons of the L-Ala groups of both glycopeptides, indicating their involvement in very strong hydrogen bonds (Table III). Relatively strong hydrogen bonding is predicted from the amide protons of the NAc groups of both glycopeptides. The temperature coefficients of the D-iGln NH_2 protons are compatible with weak hydrogen bonding. For the NAc and L-Ala amide protons, stronger hydrogen bonding is predicted for MDP than for MAP.

The solvent dependence of the chemical shifts of all amide protons of the α-anomers of MDP and MAP is illustrated in Fig. 2. Addition of increasing percentages of $CDCl_3$ to glycopeptide solutions in DMSO-d_6 produced an upfield shift of all amide resonances except that of L-Ala. These data indicate that all amide protons in MDP and MAP are fairly exposed to the solvent except the L-Ala amide proton which is involved in strong hydrogen bonding. Both temperature and solvent dependences of the amide chemical shifts are therefore in agreement.

Multiple interproton connectivities could be established from the ROESY experiments. Table IV presents the connectivities and estimated maximal distances derived from these experiments which are not automatically satisfied by the chemical structures of the glycopeptides. These values were used as constraints for the calculation of a molecular model. Two major long range ROESY connectivities in MDP were identified, namely, between the NAc CH_3 (1.80 ppm) and the L-Ala CHα (4.28 ppm) and between the D-Lac CH_3 (1.24 ppm) and the D-iGln CHγ (2.18 ppm). The latter could also be attributed to a connectivity between the L-Ala CH_3 and the D-iGln $CH_2\gamma$ in the β-anomer. This connectivity was not included in the molecular modeling calculations.

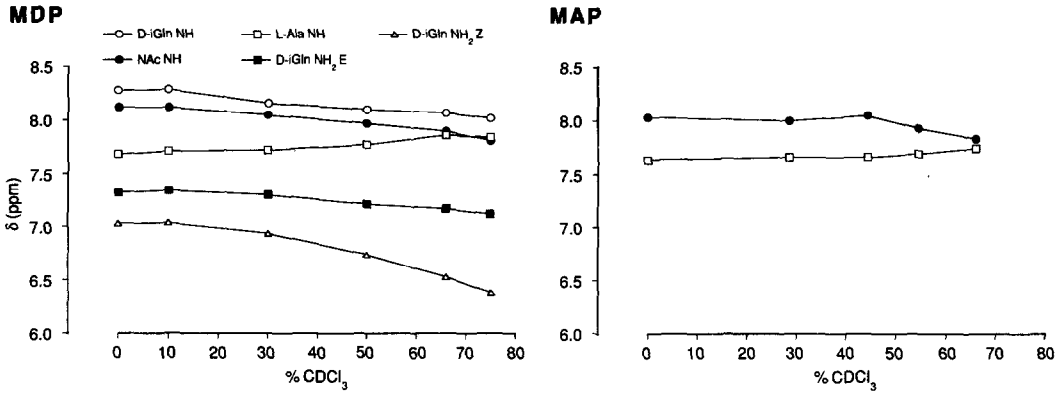


Fig. 2. Variation of the chemical shifts of the amide protons for the α-anomers of MDP (left) and MAP (right) as a function of the percentage of $CDCl_3$ added to the DMSO-d_6 solution.

In order to better characterize the three-dimensional structure of MDP, a conformational search calculation was carried out to obtain low-energy structures that were compatible with the available experimental data. Structures were obtained which were consistent with the coupling constant-derived dihedral angles, a hydrogen bond between the NAc carbonyl and the L-Ala NH, and the ROESY connectivities. The computed structures for MDP fall into several clusters of conformations shown in Fig. 3. The different

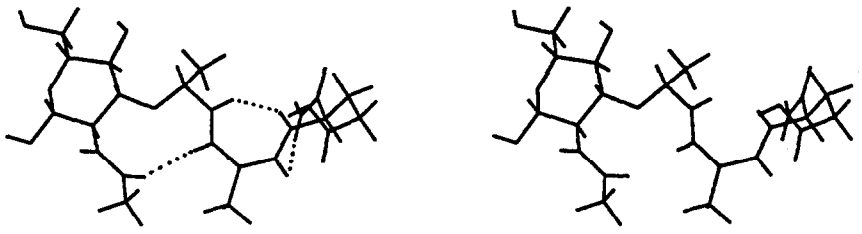
Table IV. Upper Limits of Interproton Distances Derived from ROESY Experiments and Used as Constraints for Model Calculations

Compound (anomer)	Proton(s) 1	Proton(s) 2	Maximal distance (Å)[a]
MDP (α)	NAc CH_3	L-Ala CH	5.0
	NAc CH_3	D-GlcNAc CH-1	5.0
	NAc CH_3	D-GlcNAc CH-3	5.0
	NAc NH	D-Lac CH	5.0
	D-GlcNAc CH-2	D-Lac CH	4.5
	D-GlcNAc CH-4	D-Lac CH_3	5.0
MAP (α)	NAc CH_3	L-Ala CH_3	5.5
	NAc NH	L-Ala CH_3	5.0
	D-GlcNAc CH-1	D-GlcNAc OH-6	3.5
	D-GlcNAc CH-2	D-Lac CH	3.5
	D-GlcNAc CH-3	D-Lac CH	2.5
	D-GlcNAc CH-4	D-Lac CH	3.5
	D-GlcNAc OH-4	D-Lac CH_3	5.0
	L-Ala CH	D-ProCarb CHδ	3.0
	L-Ala CH_3	D-ProCarb CHδ	5.5

[a] Distances to methyl or methylene groups are to a pseudoatom in the centroid of the protons.

A

B



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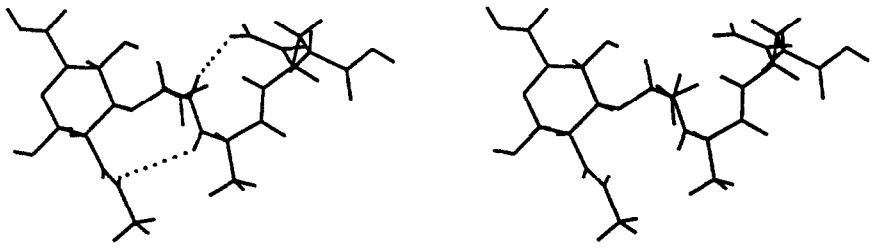

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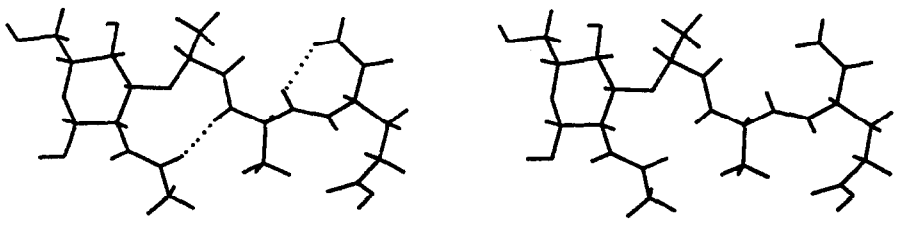

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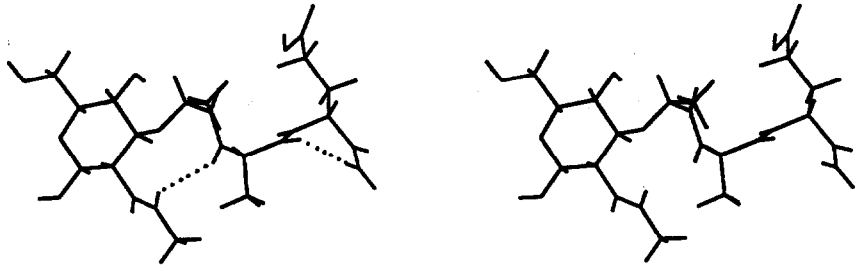

Fig. 3. Representative low energy structures of MDP obtained from a Monte-Carlo minimization algorithm using NMR-derived distance and angle constraints. (A) Superposition of 18 conformations from the lowest energy structural cluster, (B) lowest energy conformation, (C)-(E) representative conformations from other clusters. Hydrogen bonds are illustrated by dotted lines.

clusters are all similar in the first half of the molecule and differ in the hydrogen bonding patterns present the second half. Fig. 3A shows the cluster of conformations that contain the lowest energy structure found in the search. Fig. 3B shows the conformation of the lowest energy single structure present in this cluster. In this conformation, the D-iGln NH_2 is hydrogen-bonded to the L-Ala carbonyl, the D-iGln NH is hydrogen-bonded to the D-Lac carbonyl and the L-Ala NH is hydrogen-bonded to the NAc carbonyl. Figs. 3C-3E show representative structures from other clusters of higher energy (within 4 kcal/mol). In Fig. 3C, the D-Lac carbonyl is hydrogen-bonded to the D-iGln NH_2 and NH protons which is similar to the S-shaped conformation proposed previously.[7] However, this does not represent the lowest energy conformation. Figs. 3D-3E show other hydrogen bonding possibilities for the D-iGln NH_2.

The time dependences of the H-N-C-H dihedral angle of L-Ala, of the C=O··H-N distance between the L-Ala carbonyl and D-iGln NH_2, and of the C=O··H-N distance between the D-Lac carbonyl and D-iGln NH for the lowest energy conformation of MDP (Fig. 3B), as calculated by molecular dynamics, are presented in Fig. 4. The H-N-C-H dihedral angle of L-Ala fluctuates around a stable value of -75° (Fig. 4A). The C=O··H-N distance between the L-Ala carbonyl and D-iGln NH_2 fluctuates around 3Å in the first 45 ps but operates a transition to 5 Å afterwards (Fig. 4B). The C=O··H-N distance between the D-Lac carbonyl and D-iGln NH fluctuates around a stable value of 2 Å.

The low energy conformations found for MAP also clustered into several groups. Fig. 5A shows the cluster of conformations containing the lowest energy minimum found in the search. This minimum energy conformation is shown in Fig. 5B for a single structure and mimics the S-shaped conformation proposed for MDP.[7] However, the S-shaped conformation found for MAP is not due to hydrogen bonding. Other clusters of conformations with energies within 3 kcal/mol were also found and representative structures are shown in Figs. 5C and 5D.

DISCUSSION

Using interproton distances and dihedral angles derived from ^1H NMR, a molecular model could be calculated for the α-anomer of MDP. The existence of a hydrogen bond between the carbonyl group of NAc and the amino group of L-Ala, postulated in earlier studies[7,9] could be confirmed. The temperature and solvent dependences of the chemical shift of the L-Ala amine proton are supportive of hydrogen bonding between these groups (Table III and Fig. 2). However, the low-energy clusters obtained from the conformational search calculations show that a variety of hydrogen bonding patterns are possible in the second half of the MDP molecule (Fig. 3). They are all of comparable energies within a few kcal/mol of each other. The lowest energy structure found in the search is shown in Fig. 3B. It contains three well-formed hydrogen bonds: between the NAc carbonyl and the L-Ala NH, between the D-Lac carbonyl and the D-iGln NH and between the L-Ala carbonyl and the D-iGln NH_2. The fact that none of the hydrogen bonds in the second half of the molecule is observed in the temperature and solvent dependence data of the respective amide chemical shifts suggests that half of the molecule is in rapid equilibrium among two or more of these different conformations. We have carried out a preliminary 60 ps unconstrained molecular dynamics simulation (data not shown) of the lowest-energy MDP structure with solvent water molecules (Fig. 4). During the time course of the simulation, the D-iGln NH_2 hydrogen and the L-Ala carbonyl were in close proximity for about 50% of the early time period after which a transition occurred to a longer distance (Fig. 4B). The D-iGln NH hydrogen and the D-Lac carbonyl remained in close proximity almost 100% of the time at a distance compatible with hydrogen bonding (Fig. 4C). This suggests that the conformers obtained in our search are locally stable minima that the molecule samples through low-frequency (in terms of simulation time) thermal fluctuations. The S-shaped structure postulated previously on the basis of dihedral angle data seems to represent only a minor conformation in equilibrium (Fig. 3C).

The signals originating from the β-anomer of MDP are weaker than those from the α-anomer (due to its lower concentration) and often overlap the signals from the α-anomer. Therefore, an accurate determination of the molecular structure of the β-anomer could not be realized. Differences between the two anomers can however be seen from the temperature dependence of the chemical shifts of the amide protons (Table III). In the β-anomer, the temperature coefficients of protons of the NAc and L-Ala groups are both smaller than in the α-anomer, indicating stronger hydrogen bonding. In the case of the NAc amide GlcNAc sugar, as suggested previously.[7] A five-membered ring can be formed in this way for the β-anomer but not for the α-anomer. In the case of the L-Ala amide proton which forms hydrogen bonding with the NAc proton of the β-anomer, hydrogen bonding can be formed with the hydroxyl oxygen on position 1 of the D-carbonyl group, a stronger bonding exists in the β-anomer than in the α-anomer form, the latter being possibly stabilized by the NAc-OH-1 hydrogen bond.

The first hydrogen bonding in MAP (between NAc carbonyl and L-Ala NH) is observed (Fig. 5) but

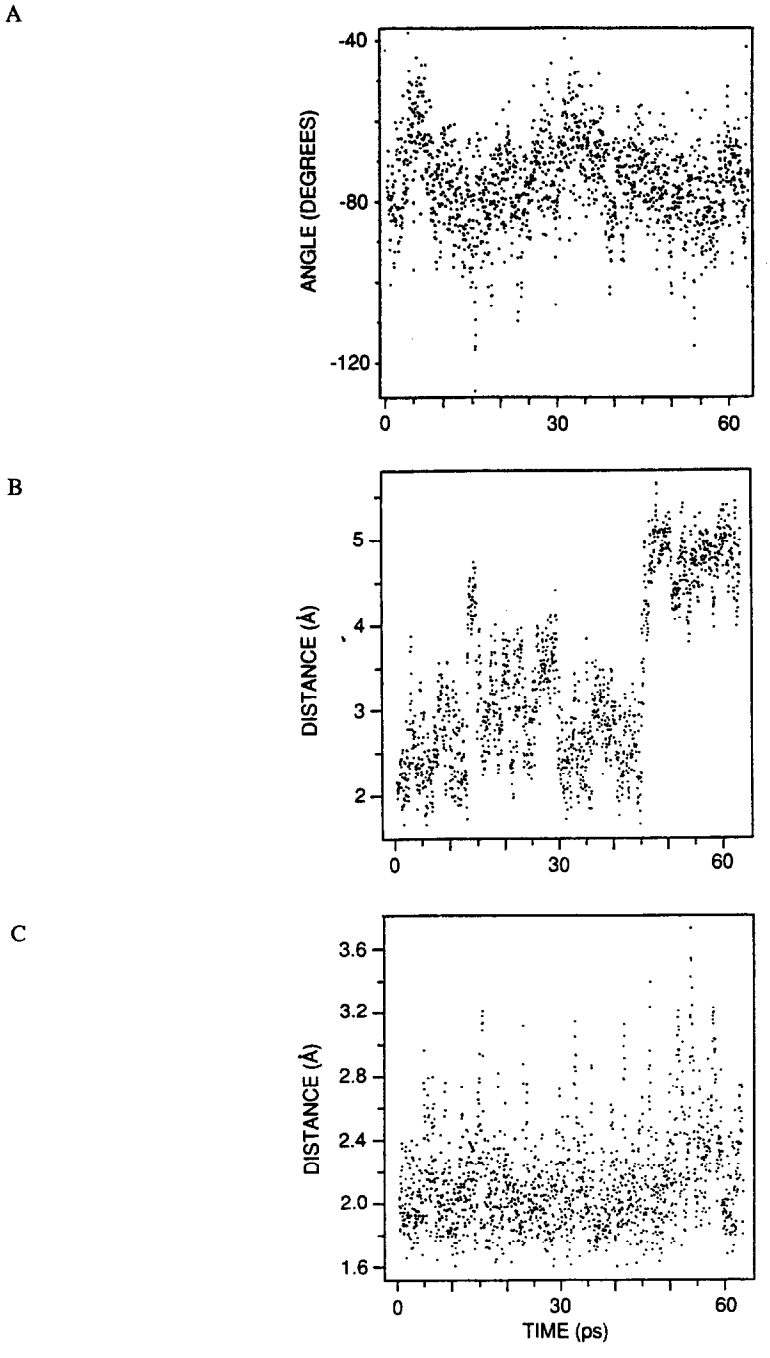

Fig. 4. Time fluctuations of the (A) H-N-C-H dihedral angle of L-Ala, (B) C=O···H-N distance between the L-Ala carbonyl and D-iGln NH_2 and (C) C=O···H-N distance between the D-Lac carbonyl and D-iGln NH for the lowest energy conformation of MDP (Fig. 3B) as calculated by unconstrained molecular dynamics.

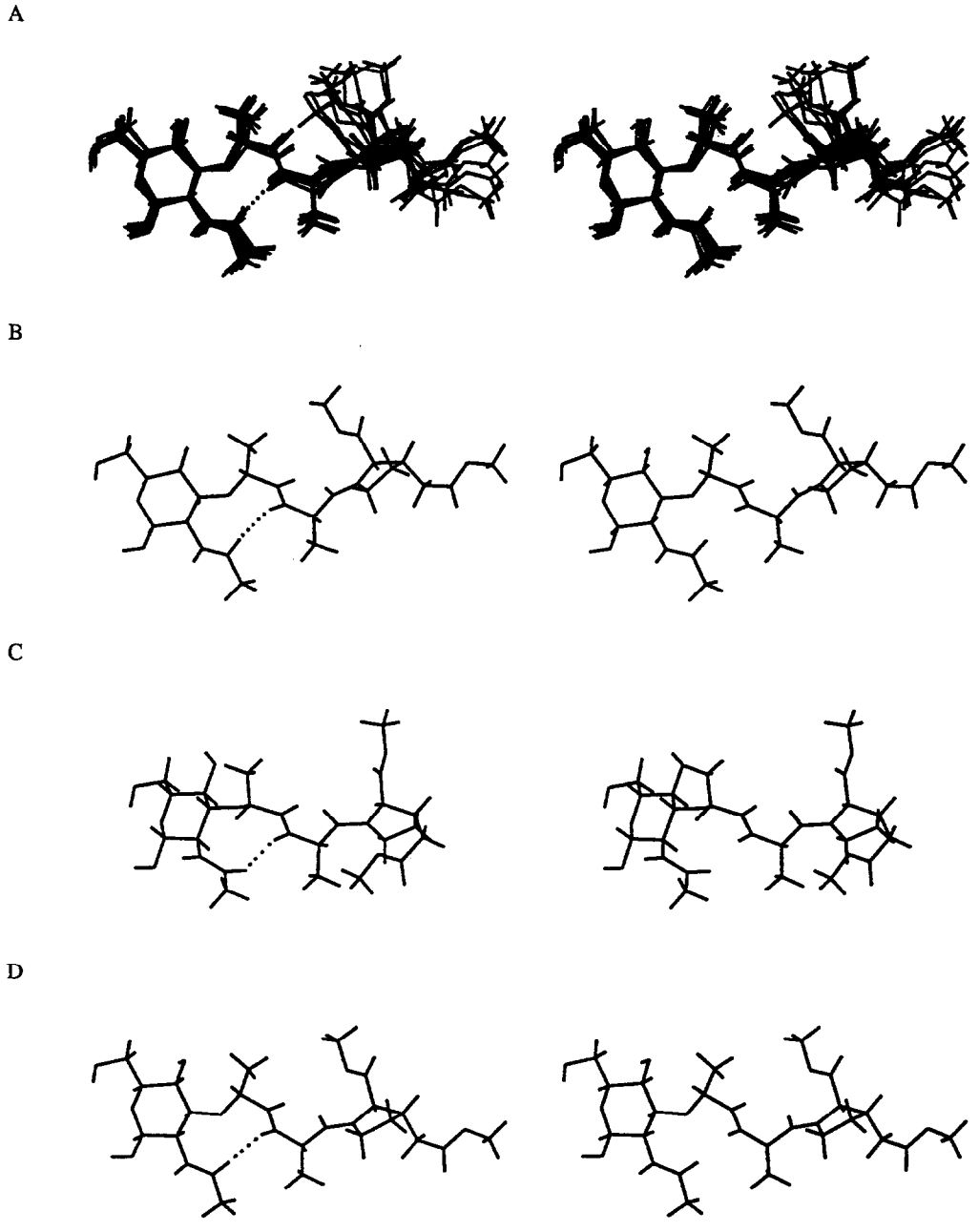

Fig. 5. Representative low energy structures of MAP obtained from a Monte-Carlo minimization algorithm using NMR-derived distance and angle constraints. (A) Superposition of 9 conformations from the lowest energy structural cluster, (B) lowest energy conformation, (C)-(D) representative conformations from other clusters. Hydrogen bonds are illustrated by dotted lines.

the temperature coefficients of the amide chemical shifts indicate a weaker bond than for MDP (Table III). The 3-carbomethoxymethyl-D-proline methyl ester part of the molecule can however adopt different clusters of conformations, the lowest energy conformation adopting an S-shaped structure.[7] Thus, the conformations of the second part of the MAP and MDP molecules are both in equilibrium between several binding possibilities but their preferred (lowest energy) structures are different.

CONCLUSION

The structural conformational search based on the NMR data has therefore confirmed the existence of a first β-turn resulting from the formation of a hydrogen bond between the NAc carbonyl and the L-Ala NH for MDP and its analogs, as reported previously.[6-8] However, the formation of a second *stable* β-turn involving hydrogen bonding between the D-Lac carbonyl and the D-iGln NH_2 in MDP is not consistent with the NMR data nor with an optimal structure of lowest energy. The lowest energy conformation of MAP was found to contain a second β-turn giving the molecule an S-shape (Fig. 5B). This compound is however biologically inactive,[12] which reflects the importance of functional *and* conformational features in the peptidic part of the muramyl peptide class of immunomodulating agents.[8,13]

ACKNOWLEDGMENT

The authors would like to thank Dr. Brian Marsden, Clinical Research Institute, Montreal for helpful discussions and experimental assistance. This work was supported by the Natural Science and Engineering Research Council of Canada and the Medical Research Council of Canada.

EXPERIMENTAL

Materials

N-Acetylmuramyl-L-alanyl-D-isoglutamine or MDP was purchased from Sigma Chemical Co. (St.Louis MO). The synthesis of N-acetylmuramyl-L-alanyl-3-carbomethoxymethyl-D-proline methyl ester or MAP has been reported.[12]

Methodology

Solutions of MDP (13 mM) and MAP (9 mM) were prepared in $(CD_3)_2SO$ for NMR measurements. Two-dimensional ^1H NMR spectra were recorded on a Bruker AM 500 (500.13 MHz) spectrometer at 293 K. Temperature-dependent one-dimensional ^1H NMR spectra were recorded on a Bruker WH 400 (400.13 MHz) spectrometer at temperatures in the range 300-350 K ($\pm$0.5 K). Complete spectral assignment was

performed using two-dimensional absolute value correlation spectroscopy (COSY) experiments. COSY spectra were transformed using a 2048 X 2048 data-point matrix and applying a sine bell window function. Interproton distance ranges were derived from rotating frame Overhauser enhancement spectroscopy (ROESY) experiments using a 250 ms mixing time and transformed using a 90° phase-shifted sine square bell function in both dimensions. ROESY spectra were transformed using a 2048 X 2048 data-point matrix. Baseline correction was carried out using the method of Ni[15]. Vicinal coupling constants were measured from one-dimensional spectra having been resolution enhanced using a Lorentzian-to-Gaussian function.

The conformational space available to the MDP and MAP molecules was explored using a Monte-Carlo minimization algorithm (Purisima, E.O.; Hogues, H.; Ripoll, D.R., manuscript in preparation) implemented as an SPL (SYBYL programming language) program in the molecular modeling program SYBYL 5.4 (Tripos Assoc., Inc.). The Tripos force-field was used with partial charges obtained using the Pullman method.[16] Non-bonded cutoffs were turned off and a distance dependent dielectric constant was used. Distance upper bounds were derived from the ROESY data and used as half-harmonic restraints with a force constant of 5.0 kcal/mol in the potential energy function. Distance upper bounds to methyl or methylene groups were with respect to pseudo-atoms located at the centroid of the methyl or methylene hydrogen atoms. Coupling constants were converted to harmonic dihedral angle restraints (in degrees) with a force constant of 0.01 kcal/mol. The coupling constant data of Fermandjian *et al.*[7] for D-iGln were also used. A distance constraint upper bound of 2.75 Å was added for the NAc carbonyl and the L-Ala NH pair. One thousand energy minimizations were performed for both MDP and MAP.

Molecular dynamics simulations were performed the SYBYL 5.4 program. A time step of 1.5 fs was used with all bonds constrained to their equilibrium value using the SHAKE algorithm. The simulation was operated at 300 K with a thermalization period of 3 ps using a temperature bath method with a coupling time of 1.2 ps. The molecule was immersed in a 15 Å sphere of water molecules centered around the second half of the molecule. All calculations were done on a Silicon Graphics 4D-280 computer.

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