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^{1}H-N.M.R. STUDIES OF A NATURAL IMMUNOADJUVANT PEPTIDOGLYCAN MONOMER: PROPOSED STRUCTURE IN SOLUTION IN METHYL SULFOXIDE

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ABSTRACT

The conformation in solution in methyl sulfoxide of the immunoadjuvant peptidoglycan monomer (PGM), obtained by digestion with lysozyme of the linear peptidoglycan polymer isolated from *Brevibacterium divaricatum*, was studied by ^{1}H-n.m.r. spectroscopy. The temperature dependence of the chemical shift of the resonances of the amide protons suggested that the amino group of alanine-5 is involved in hydrogen bonding, most probably to the α-carbonyl of the isoglutamine which showed restricted rotation, as indicated by the large chemical shift non-equivalence for the resonances of the βCH_2 group. A cyclic structure is proposed for the *C*-terminal pentapeptide of PGM, which is further supported by various n.O.e. interactions involving the *meso*-diaminopimelic residue and the *N*-acetylmuramoyl group.

INTRODUCTION

Peptidoglycan, a component of the cell walls of Gram-positive and Gram-negative bacteria, has various biological activities[1]. The peptidoglycan and synthetic analogues of lower molecular weight exhibit significant immunomodulating properties in mammals[2–5].

The peptidoglycan monomer (PGM) obtained by digestion of the polymer with lysozyme has been identified as {2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-ethyl-1-carbonyl]-D-glucopyranose}-L-alanyl-D-isoglutaminyl-[(L)-*meso*-diaminopimeloyl-(D)-amido-(L)-(D)-alanyl-D-alanine][6–9].

It is easy to prepare the smaller fragments in pure form, and their structures are sufficiently simple to permit chemical modification in order to achieve optimal pharmacological properties. A prerequisite for such work is an understanding of

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the conformation of the immunomodulating peptidoglycan fragments. This was a reason for previous ^{1}H-n.m.r. studies of such PGM analogues as *N*-{2-acetamido-2-deoxy-3-*O*-[(*R*)-ethyl-1-carbonyl]-D-glucopyranose}-L-alanyl-D-isoglutamine (*N*-acetylmuramoyldipeptide, MDP)[10–13], {2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-ethyl-1-carbonyl]-D-glucopyranose}-L-alanyl-D-isoglutamine (GMDP)[14], the tetrapeptide N^2-(L-alanyl-D-γ-isoglutaminyl)-N^6-glycyl-LL-2,6-diaminopimelic acid, and the pentapeptide N^2-(L-alanyl-D-γ-glutamyl)-N^6-glycyl-LL-α-2,6-diaminopimeloyl-D-alanine[15], which indicated the existence of non-random structures in solutions in water or methyl sulfoxide.

We now report a ^{1}H-n.m.r. study of PGM, which is an apyrogenic molecule in contrast to MDP.

EXPERIMENTAL

Preparation of peptidoglycan monomer. — PGM was obtained by digestion with lysozyme of the linear non-cross-linked peptidoglycan polymer chains isolated from culture fluids of penicillin-treated *Brevibacterium divaricatum*, and purified by molecular sieving (Sephadex and Biogel)[6,7].

N.m.r. spectroscopy. — A 15mM solution of PGM in $(CD_3)_2SO$ (6 mg in 0.4 mL) was used, and the spectra were referenced to residual Me_2SO (2.51 p.p.m.).

The 1D spectra (500 MHz) were obtained with a Bruker AM-500 spectrometer at 25–50°. Typically, each spectrum was collected using a single pulse of 90°, a relaxation delay time of 3 s, and a total sweep width of 5000 Hz sampled with 16 384 points (16 k). Homonuclear gated decoupling was utilized during the relaxation delay in order to suppress resonance of the solvent. Data processing employed a Lorentz line-broadening factor of 0.6 to 1.0 Hz.

The 2D spectra (360 MHz) were obtained with a modified Bruker HXS-360 spectrometer equipped with a Nicolet 1180 data system and GENT-1180 software. COSY[16,17], relayed COSY[18,19], (NOESY)[20,21], 2D *J*-resolved[22], and double-quantum 2D spectroscopy for protons[23] were performed at 35 ±2°. The COSY and NOESY spectra were obtained in the phase-sensitive and magnitude modes. The COSY, relayed COSY, and NOESY experiments had 2048 points in the F2 dimension and 256 slices in the F1 dimension, which were zero-filled to 1024 points. Each slice was obtained using 64 averages, a relaxation delay of 4 s, and a spectral width of 3310 Hz. The resolution in the F2 domain was 3.3 Hz/point and, in the F1 domain, 6.6 Hz/point. The NOESY eperiment employed a mixing time of 500 ms. In the relayed COSY experiment, the delay for relay transfer of magnetization was adjusted to 30 ms in order to optimize sensitivity for the coupling constants of interest (~8 Hz). The 2D *J*-resolved experiment was performed with 8192 points in the F2 dimension and 64 points in the F1 dimension, which were zero-filled to 256 points. The frequency ranges were 3310 Hz for the F2 dimension and 40 Hz for the F1 dimension. Each slice contained 16 accumulations with a relaxation delay of 4 s.

The double-quantum 2D experiment for protons was obtained with a sweep

width of 3310 Hz in the F2 dimension and 6620 Hz in the F1 dimension. The double-quantum coherence pulse was set to 135°. The number of points in both dimensions, the zero-filling, the pulse recovery time, and the number of averages per slice were the same as in the COSY experiment.

RESULTS AND DISCUSSION

A fresh solution of PGM in methyl sulfoxide contains mainly the α anomer

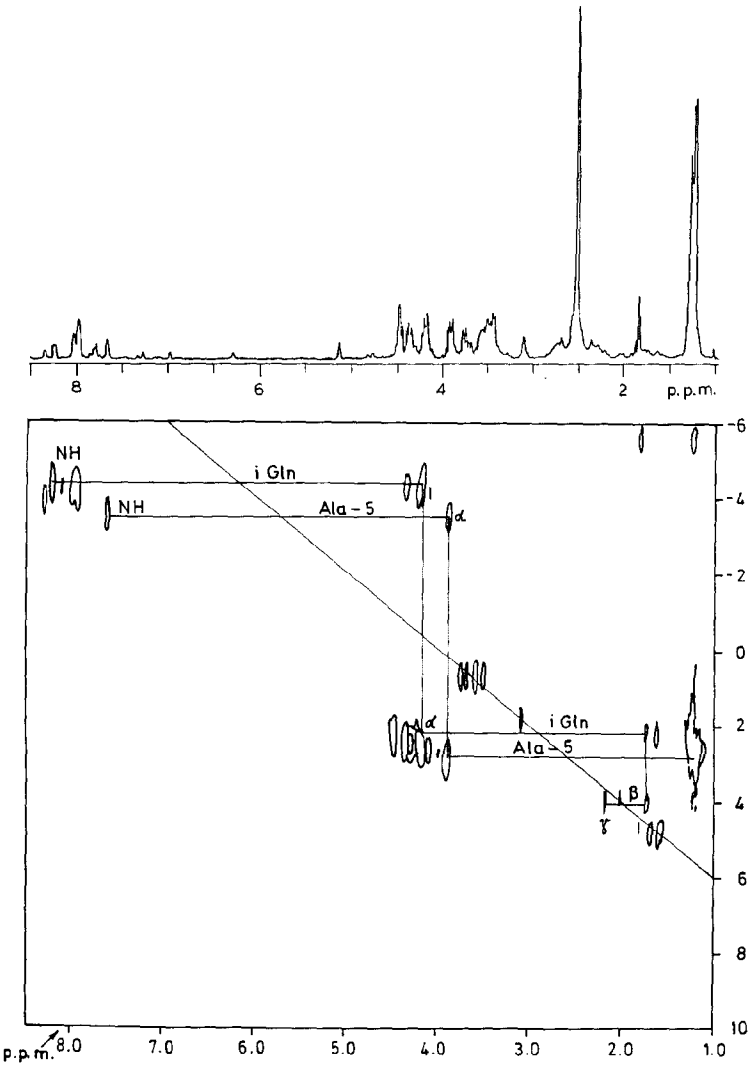

Fig. 1. Double-quantum 2D ^1H–^1H spectrum of the peptidoglycan monomer in solution in $(CD_3)_2SO$ at 25°: chemical shifts referenced to internal Me_2SO.

TABLE I

CHEMICAL SHIFT DATA (δ IN P.P.M.) OF THE PEPTIDOGLYCAN MONOMER (PGM) IN SOLUTION IN $(CD_3)_2SO$ AT 25°

Residue	*Proton (s)*	δ	*Residue*	*Proton (s)*	δ
GlcNAc	H-1	4.75	L-Ala	NH	7.93
	H-2	3.49		αCH	4.31
	H-3	3.75		βCH	1.26
	H-4	n.r.[b]			
	H-5	3.12	D-iGln	NH	8.20
	H-6	n.r.		αCH	4.16
	H-6′	n.r.		βCH	1.72
	NH	8.29		β'CH	2.01
	methyl	1.82		γCH	2.19
	OH-3	5.23		NH (*E*)[c]	7.29
	OH-4	n.r.		NH (*Z*)	6.94
	OH-6	n.r.			
			meso-A_2pm	NH	7.98
MurNAc	H-1	5.10		αCH	4.19
	H-2	3.41		βCH	1.62
	H-3	3.08		γCH	1.35
	H-4	3.75		γ'CH	1.42
	H-5	3.69		β'CH	1.62
	H-6	3.60		α'CH	4.19
	H-6′	3.69		NH	7.98
	NH	7.76		NH (*E*)	7.95
	methyl	1.81		NH (*Z*)	7.22
	αCH lac[a]	4.45			
	βCH lac	1.27	D-Ala	NH	7.94
	OH-1	6.30		αCH	4.17
	OH-6	n.r.		βCH	1.22
			D-Ala	NH	7.59
				αCH	3.88
				βCH	1.21

[a]lac = lactoyl group. [b]Not resolved. [c]*E, Z* amide protons are *anti* and *syn* to the oxygen atom of the adjacent carbonyl group.

with respect to the *N*-acetylmuramoyl (MurNAc) residue. The peaks of the β anomer (<10%) were neglected. COSY, relayed COSY, and 2D double-quantum experiments allowed the unequivocal assignment of all proton signals of the pentapeptide moiety, most of the sugar ring protons, and two of the five hydroxyl protons in the amino sugar residues. In the double-quantum 2D ^1H–^1H spectrum of PGM (Fig. 1), the assignments of the Ala-5 and isoglutamine (iGln) protons are given as an example. Each of the direct connectivities NH→α-H and α-H→β-H for both amino acid moieties, and β-H→γ-H for the iGln residue was manifested by a pair of signals that were symmetrical with respect to the skew diagonal at the ω_2 position of the two interacting spins. The chemical shift data are given in Table I.

The $\Delta\delta$ value for the iGln βCH_2 protons was 0.29 p.p.m. (Table I), which is substantially larger than that (0.09 p.p.m.) for protons of glutamic acid or iso-

glutamate in small peptides[26], as observed also for MDP[10,11]. A similar, although smaller effect (0.07 p.p.m.) was observed for the γCH_2 protons of *meso*-diaminopimelic acid (A_2pm). These effects indicate restricted rotation of the side chains of the two adjacent amino acid residues which, however, appears to be much more severe for iGln. This finding is consistent with the assumption that iGln is "immobilized" by hydrogen bonding.

The chemical shifts of the resonance of the amide protons varied linearly in the temperature range 25–50°. The temperature-dependence coefficients (Fig. 2) are in the range -9.6×10^{-3} to 1.3×10^{-3} p.p.m./degree. The latter coefficient was exhibited by the amide proton of Ala-5, indicating either involvement in a hydrogen bond or inaccessibility to the solvent molecules. A Dreiding model of PGM strongly favoured the former possibility with the acceptor being the iGln α-carboxamido group. This conclusion is supported by the large $\Delta\delta$ value for the iGln βCH_2 protons, as discussed above. The Dreiding model also suggests that the non-equivalence of the resonance of the A_2pm γCH_2 protons may arise from the closer proximity of one to the Ala-4 NH. However, it is also possible that the restricted rotation of the hydrogen-bonded iGln moiety is extended, to some extent, through the peptide backbone, to the adjacent A_2pm residue.

In order to obtain further insight into the conformation of PGM, the C-3–C-2–NCO dihedral angles (θ) were derived from the $^3J_{H,NH}$ values in accordance with published relationships[24,25], and the possible values are given in Table II. The experimental error of the measured J values was ±0.2 Hz, which resulted in an error of ±2° in derived dihedral angles. The J values were temperature independent in the range investigated. Comparison of MDP[13] and PGM shows that the dihedral angles in the MurNAc and iGln residues are identical, whereas those in the L-Ala moiety differ (PGM −88, −152; MDP −81, −159). This situation appears to be a consequence of the different pattern of hydrogen bonding (Ala-5→iGln in PGM; Ala-1→MurNAc in MDP)[13,14] imposed by elongation of the peptide chain.

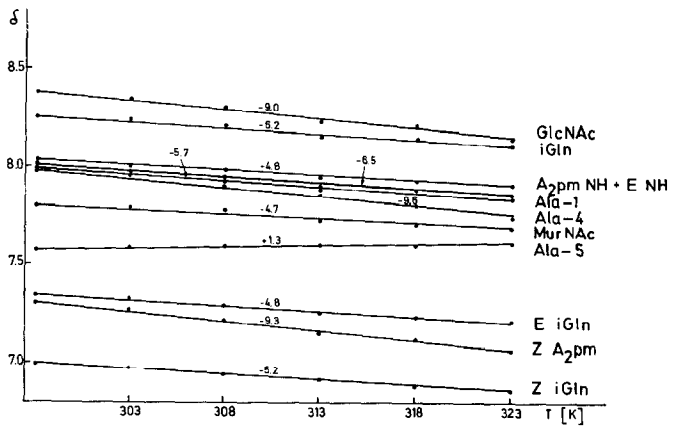

Fig. 2. Temperature dependence of the chemical shifts of the resonances of the amide protons of peptidoglycan monomer (15mM) in $(CD_3)_2SO$.

TABLE II

$^3J_{H,NH}$ (Hz) FOR THE PEPTIDOGLYCAN MONOMER (PGM) AND THE CORRESPONDING DIHEDRAL ANGLES (θ)[a]

Residue	*Proton*	J	θ
GlcNAc	NH	8.4	−92, −148
MurNAc	NH	7.9	−86, −154
L-Ala	NH	8.0	−88, −152
D-iGln	NH	8.0	88, 152
	NH (*E*)	3.4	
	NH (*Z*)	3.2	
meso-A_2pm	NH	7.6	85, 155
	NH (*E*)	n.o.[b]	
	NH (*Z*)	3.2	
D-Ala (Ala-4)	NH	7.2	82, 158
D-Ala (Ala-5)	NH	6.4	78, 162
GlcNAc	H-1	6.8	
MurNAc	H-1	2.6	
L-Ala	αCH	7.2	
	βCH	7.2	
D-iGln	αCH	7.2	
	βCH	6.2	
	β'CH	7.8	
	γCH	8.0	
meso-A_2pm	αCH	7.4	
	βCH	7.2	
D-Ala (Ala-4)	αCH	7.4	
	βCH	7.4	
D-Ala (Ala-5)	αCH	6.4	
	βCH	7.2	

[a]See refs. 24 and 25. [b]Not observed.

The geometry of the D-lactoyl group cannot be established easily from the ^{1}H-n.m.r. data because of the lack of vicinal protons. However, X-ray analysis has shown[27,28] that this group adopts dihedral angles of ~90° and ~ −10° for ϕ and ψ, respectively.

Fig. 3 presents the intramolecular proton–proton connectivities deduced from the respective n.O.e. values observed in the NOESY contour plot for PGM in solution in methyl sulfoxide, and the amide part is given in Fig. 4. The observed connectivities indicate that the following non-bonded groups in PGM are closer than 0.45 nm; the α' amino group of A_2pm and αCH of the D-lactoyl residue, αCH of A_2pm and H-2 of the MurNAc residue, and αCH of A_2pm and the amide proton of the MurNAc residue. Although the expected n.O.e. connectivity between αCH of Ala-4 and NH of Ala-5 was not observed, the two D-alanine spin systems were distinguished by different titrimetric properties[9].

As far as the connectivities between the muramoyl and the peptide moieties are concerned, most of the data in this study (Fig. 3) are similar to those determined for GlcNAc-(1→4)-MurNAc-L-Ala-D-isoGln[14]. Thus, there are several spatial restrictions on the structure of the pentapeptide and the disaccharide moieties.

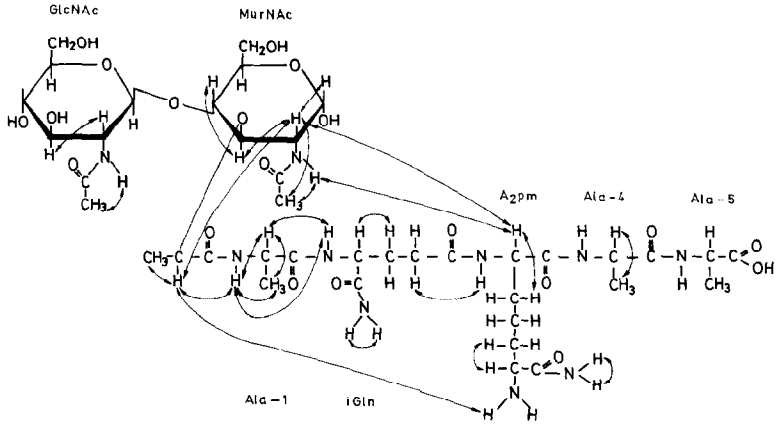

Fig. 3. Structure of peptidoglycan monomer in solution in $(CD_3)_2SO$ and deduced from n.O.e. contacts.

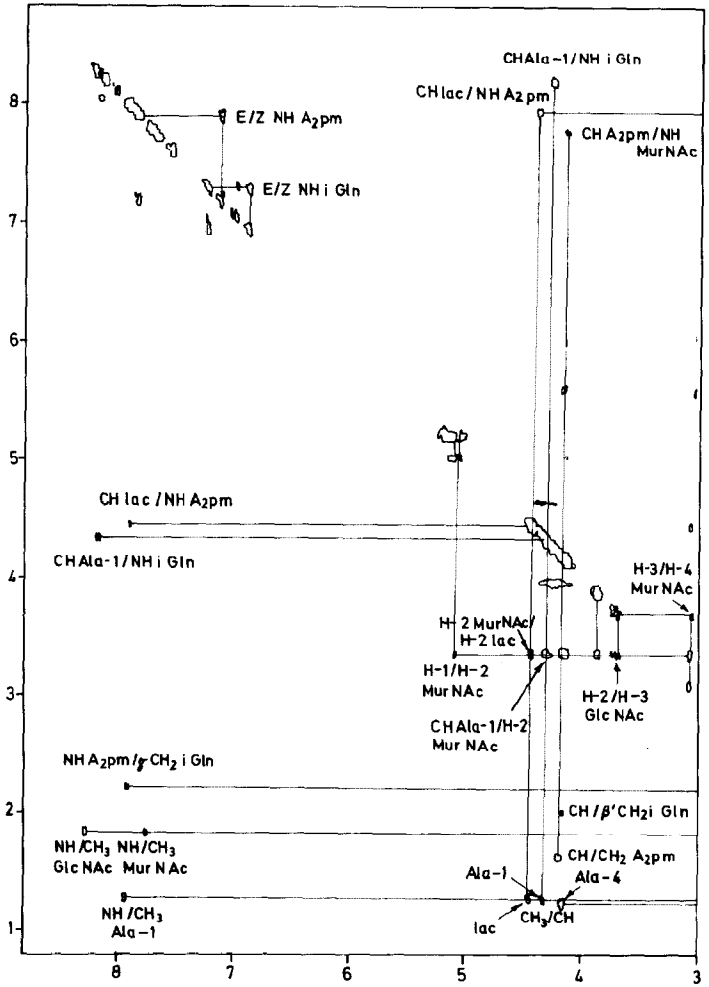

Fig. 4. NOESY contour plot spectrum (magnitude mode) of peptidoglycan monomer in solution in $(CD_3)_2SO$ (amide part of the spectrum).

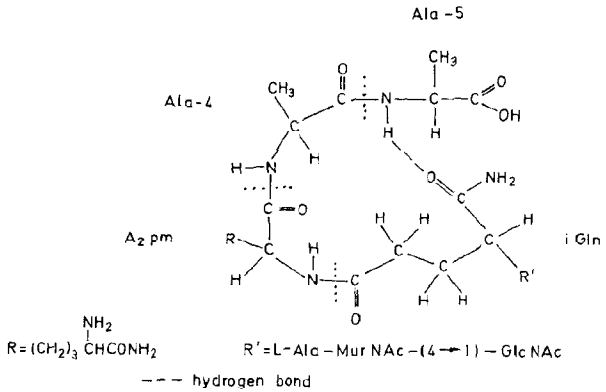

Fig. 5. Possible structure of peptidoglycan monomer stabilized by a hydrogen bond involving the amide proton of alanine-5 and the α carbonyl group of the isoglutamine residues.

N.O.e. data, taken tegether with the hydrogen-bonding data obtained by temperature dependence studies and the calculated values of $\phi \sim -70°$ and $\psi \sim -130°$ for GlcNAc-(1→4)-GlcNAc[29,30], suggest that the projected structure of the pentapeptide moiety of PGM is as shown in Fig. 5, and that the disaccharide moiety lies below the plane of Fig. 5. These two components of PGM appear to form "globular" structure, as indicated by the n.O.e. connectivities between the A_2pm and MurNAc protons shown in Fig. 3. Metal-ion chelating properties would be expected for a molecule of the structure proposed and, indeed, they are pronounced[7,31].

Thus, the dihedral angles, temperature coefficients, and 2D n.m.r. data can be interpreted in terms of a folded structure for PGM stabilised by an intramolecular hydrogen bond.

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