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Solution Conformation of the Immunomodulator Muramyl Dipeptide: Restrained Molecular Dynamics Based on Nuclear Magnetic Resonance Data and Semiempirical MO Study

Viljem Harb[+], *Janez Mavri*[*], *Jurkica Kidrič*[*] *and Dušan Hadži*[+*]

[+]*Lek – Pharmaceutical and Chemical Works, Ljubljana*
[*]*Boris Kidrič Institute of Chemistry, P.O. Box 30, 61115 Ljubljana, Yugoslavia*

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Conformational analysis of *N*-acetyl-muramyl-L-alanyl-isoglutamine (MAG) was preformed by molecular dynamics. Initial geometries were generated by random setting of dihedral angles. In order to narrow the vast conformational space of MAG, experimental NOE connectivities and connectivities based on hydrogen bonds[1] were included in the potential energy function. The energy differences between seven structures with the lowest total energy are surprisingly small. The rank order of energy minimized structures yielded by the semiempirical MO method AM1 is not the same as predicted by molecular mechanics, but the differences in energies are very small. The S-shaped conformation suggested by NMR analysis[1] seems to be possible according to the energy criterion.

INTRODUCTION

N-acetyl-muramyl-L-alanyl-D-isoglutamine (MAG) is the oldest and the most extensively studied representative of immunomodulating muramyl peptides (MP) derived from bacterial cell wall. Very recently, the activity of MAG on human immunodeficiency virus has been observed *in vitro*.[2] Numerous analogues have been synthesized and tested[3,4] in the search for more specific activity. Although discussions about the location of the MP receptor are not conclusive, it is at least fairly certain that the mechanism of action of MP involves specific receptors.[5,6] The quest for bioactive conformation, which is intimately connected with receptor binding, becomes very complex in the case of small peptides as ligands. In view of the very large conformational space available to linear oligopeptides, it is quite legitimate to question[7] the possibility of delineating anything like bioactive conformation.

Nevertheless, it is possible that, the circumstances given by the primary structure, even dipeptides may assume a limited number of low energy conformations which may be close to the conformation fitting to the receptor binding site. Recent NMR confor-

mational analyses of MAG and its butyl ester, murabutide, suggest that at least some preferential conformations in dimethylsulfoxide (DMSO) solution do exist.[1,8]

Conformation of MAG in DMSO solution was studied by NMR methods by Chapman *et al.*[9] and, more extensively and along with its butyl ester and D-alanine isomer, by Sizun *et al.*[1] and Fermandjian *et al.*[8] The investigation of Chapman *et al.* was limited to the assignment of proton signals and the determination of temperature coefficients of amide NH shifts whereas the latter work[1,8] included determination of the coupling constants and connectivities derived from 2D experiments. Fermandjian and coworkers[1,8] have proposed for MAG an S-shaped structure composed of two adjacent β II turns. The first of them is stabilized by a hydrogen bond between AlaNH and the acetamido carbonyl (H-bond 1 henceforth) whereas the other β turn includes the H-bond formed by α-carboxamide of D-iGln and the d-Lac carbonyl (H-bond 2 henceforth). The NH temperature coefficient related to H-bond 1 is definitely in the intramolecular range, in agreement with the results of Chapman *et al.*[9], whereas the temperature coefficient of H-bond 2 is -5.30[1] and is concentration dependent.[9] Nevertheless, Sizun *et al.*[1] accept the intramolecular H-bond 2 on the ground of coupling constants, indicating the existence of the second β II turn.

The S-shaped structure proposed on the basis of NMR data[1,8] is probably not the only one to satisfy the experimental data and it is unlikely that such a small peptide would exist in solution in a single conformation. We have, therefore, decided to explore the conformational space of MAG by molecular dynamics (MD). In view of its many degrees of freedom, systematic exploration would be virtually impossible without including constrains obtainable from NMR experiments. Particularly useful in this respect are the nuclear Overhauser effect (NOE) and the NH temperature coefficients. Inclusion of the NOE information in the potential energy function brings in, besides constraining the conformational space, the entropy effects as well as the influence of the solvent on some part of the conformational space. The calculated energies thus represent the free energies of various structures and can be related to their populations.

In addition to MD calculations, we have performed energy minimizations, using the semiempirical AM1 scheme, of three low energy conformations of MAG as yielded by MD. This can be considered as a check of the quality of the force field used in MD calculations.

MD calculations produced a large number of low energy conformations, some of which can be assembled to structurally similar families. The one with the lowest energy does not include the model suggested by Fermandjian and coworkers.[1,8] The lowest energy structures are more open due to the elongation of H-bond 2. A structure that is more similar to that proposed by experimentalists[1,8] was obtained by quenching. Its energy is somewhat higher, but its occurrence in the equilibrium mixture is quite possible.

METHODS

Atom and residue labelling of MAG is shown in Figure 1. The starting conformation of MAG was obtained by model building using the program INSIGHT[10] for interactive molecular graphics and molecular modeling, implemented on the Silicon Graphics SG4D50GT workstation. Ten additional starting conformations were generated by randomly varying all dihedral angles in the molecule. The muramyl fragment was set in the α anomeric form.

For molecular simulations of MAG (molecular dynamics and energy minimization), the program DISCOVER[10] was used; nonbonding and bonding parameters were taken from the standard Hagler-Lifson force field.[10].

The potential energy function $V(r)$ consists of the usual terms representing bond-angle bending, harmonic (out of plane, out of tetrahedral configuration) dihedral bending, sinusoidal dihedral torsion, van der Waals and electrostatic (Coulomb) interactions. In order to satisfy the distance constraints (NOE's and hydrogen bonds) extra terms were added to the potential energy function :

$$V(r) = \frac{1}{2} K\,(2r - \max - x)\,(x - \max) \qquad \text{if} \quad r < x$$

$$V(r) = \frac{1}{2} K\,(\min - r)^2 \qquad \text{if} \quad r < \min$$

$$V(r) = 0 \qquad \text{if} \quad \min < r < \max$$

$$V(r) = \frac{1}{2} K\,(r - \max)^2 \qquad \text{if} \quad r > \max$$

$$V(r) = \frac{1}{2} K\,(2r - \min - y)\,(y - \min) \qquad \text{if} \quad r > y$$

where r is the distance between the restrained atoms, min and max are their experimentally determined minimum and maximum distances, respectively. The x and y values represent the range of the restraining potential where parabolic behaviour is replaced by linear in order to avoid the prohibitive larger forces that would occur at large violations.

Since in preliminary test calculations the lower bound value was never violated, the lower bound restraining terms were omitted and upper bound restraining terms were set to 3.5 and 2.3 Å for NOE distances and H-bonds, respectively. In all MD simulations, the force constants in the restraint terms for hydrogen bonds and NOE distances were set to 50 and 10 kcal mol^{-1} Å^{-2}, respectively. A typical MD of 40 ps was preceded by 200 steps of the steepest descent energy minimization.

Leap-frog algorithm was used with a time step of 1 fs to integrate the equations of motion. The MD was carried out with coupling to a bath at constant temperature

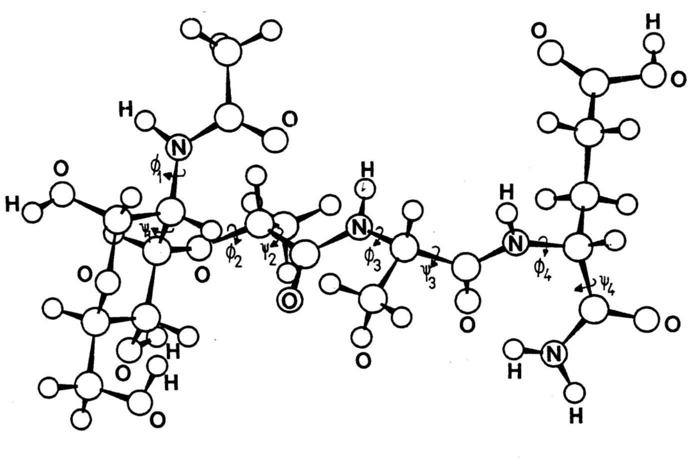

Figure 1. Atom numbering and main torsional angles of MDP.

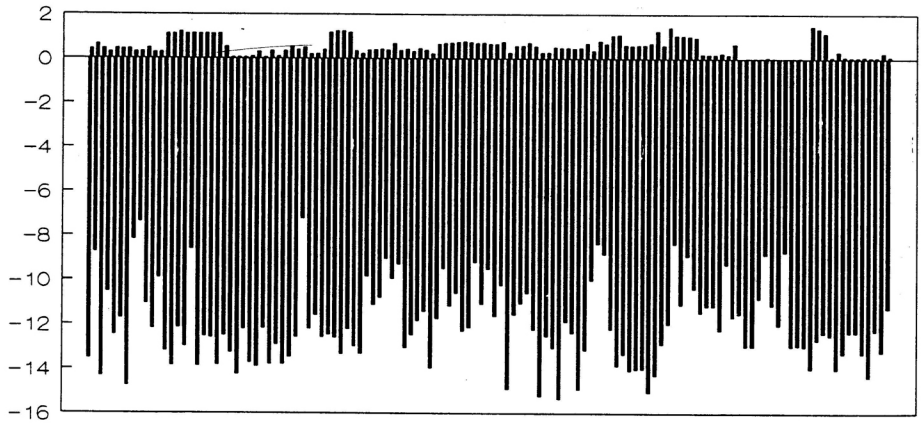

Figure 2. Conformation space of MDP
Upper part of the diagram: Sum of the NOE and hydrogen bonds distance constraints violations in Å. Lower part of the diagram: Total DISCOVER energy in kcal/mol. The restraining part of the potential is not included.

of 300 K. Coordinates were stored every 2000 steps and kinetic energy was removed later by the va09a (adopted basis Newton-Raphson)[11] energy minimization with the requirement that maximum force be less than 0.05 kcal mol^{-1} $Å^{-2}$. Eleven MD runs were performed starting with different initial geometries generated by random setting of dihedral angles in order to explore parts of the conformational space as large as possible. This was necessary since the MD trajectories animation revealed that the molecule did not undergo major conformational changes in the course of dynamics, *i. e.* the dihedral angles determining the conformation of the main chain did not change significantly. In order to determine the influence of the distance restraints on the total energy, a structure with strongly violated constraints and with both hydrogen bonds stabilizing β II turns broken was created by means of interactive computer graphics. The structure was subject to energy minimization without any distance restraints.

In order to test the reliability of the molecular mechanics force field, the semiempirical molecular orbital method AM1 was applied, since the size of the system prevents application of the *ab initio* methods even with minimal basis sets and omission of geometry optimization. Geometry optimizations without any geometrical restraints were done on the AM1 level[2] with the program AMPAC.[13] Option PRECISE was specified.

Three structures were optimized at the AM1 level. Starting points for two of them were DISCOVER structures; the first had the lowest total energy, the second included two β II turns, as proposed by Sizun *et al.*, and the third was the »extended« DISCOVER structure (*vide infra*).

RESULTS

Superposition of seven conformations with the lowest total energy obtained by quenching, using the forcing potential of the form described above, is shown in Figure 3a. The structures correspond to energies with sequence numbers 3, 7, 66, 71, 74, 77 and 88 in Figure 2.

A brief inspection of Figure 2 suggests that the conformational space explored is quite complex and the energy differences between the various minima are surprisingly small. The agreement with experimentally determined connectivities is also good; in all the cases, the sum of distance violation is below 2. In other words, many conformations satisfy the experimental constraints. In the best of »best« structures as depicted in Figure 3a, the sum of distance violation is even below 0.5 Å and one could say that the set does not contradict the experimental information. The corresponding dihedral angles and H-bond characteristics are collected in Table I.

Figure 3a shows that the structures with low DISCOVER energies differ from the one suggested by Sizun *et al.*.[1] The structure that is most similar to the experimentally suggested one, obtained by MD quenching, is shown in Figure 3b. In the former case the S-shaped conformation is not reproduced and elongation of both intramolecular hydrogen bonds, suggested by experiment, occurs.

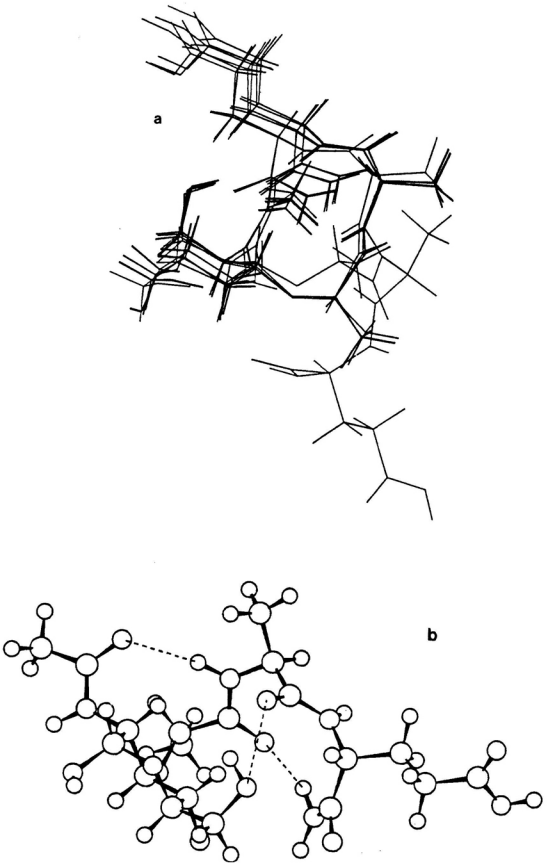

Figure 3 a: Superposition of seven distance restrained energy structures with the lowest total energy. b: The most similar structure, as obtained by MD quenching, to the one suggested by experiments.

TABLE I

Dihedral angles and hydrogen bond

	N-acetyl(a)		d-Lac		L-Ala		D-Glu		*r*1*	*r*2**
	$\varphi 1$	$\psi 1$	$\varphi 2$	$\psi 2$	$\varphi 3$	$\psi 3$	$\varphi 4$	$\psi 4$		
1	60	56	58	36	47	57	81	–77	2.74	2.71
2	61	56	59	37	47	57	81	–77	2.76	2.76
3	59	56	51	40	46	57	86	–62	2.66	2.58
4	59	56	51	39	46	57	85	–64	2.68	2.63
5	61	56	58	36	47	56	79	–80	2.78	2.82
6	61	56	59	36	47	57	79	–79	2.76	2.77
7	55	57	137	–110	–77	84	91	52	2.99	2.38

* distance H–O in the first β II turn in Å
** distance H–O in the second β II turn in Å

In addition, one can conclude from the poor overlap of the isoglutamine side chains on the superposition scheme (Figure 3a) that this side chain exhibits much greater mobility that the rest of the molecule and this was confirmed by MD trajectory animation. Exclusion of experimental information on the hydrogen bonds from restrained MD significantly increases the mobility of the molecule.[14]

It is important that the sigmoid structure similar to that suggested by experiments should possess only 2.9 kcal mol^{-1} higher DISCOVER energy than the lowest energy one. Since the distance violations in both cases are small, we suggest that the structure proposed by Sizun *et al.*[1] is accessible, although it is not the only possible one consistent with the experimental connectivities.

DISCUSSION

There is a general belief that peptides constituted by less than ten amino acid residues lack unique conformation in solution. In the case of MAG, the results of calculations based on experimental data suggest that this may be an exaggeration and that, under the circumstances, certain families of conformations may be dominant.[15]

The procedure of choosing the starting point(s) for exploring the conformational space of the MAG might, in principle, be conducted in a more conventional way by using the distance geometry algorithm.[16] We believe that this would not significantly improve the results, since eleven starting points chosen at random were used. The MD samplings of total 40 ps should be sufficiently long to explore the relevant parts of the conformational space, consistent with the distance restraints. Nevertheless, we are aware that four of the seven »best« conformations were produced within one cut of twenty other MD runs. It should be born in mind that the global minimum was hardly accessible from the residual MD runs and it is quite possible that the global minimum is separated by a high barrier from the rest of phase space. It is, however, important that the sum of distance violations of practically all of the energy minimized structures turns out to be small, as shown in Figure 2. In such a case, the application of time dependent NOE constraints[17] is not likely to improve the MD sampling procedure. If a higher bound distance value and/or lower values for the force constants in the distance restraint terms were used, the sum of distance violations would be decreased as well as the energy gap between the conformations.

Even so, the energy differences are very small, allowing for the existence of a variety of conformations with energies of only 1 kcal mol^{-1} above the seventh, energetically most favourable structure. The conformation chosen might be an artefact of the applied force field. Comparison of the AM1 and DISCOVER calculated energies in Figure 5 reveals that the former method favours the extended structure C over A in contrast to DISCOVER, which predicts B to be the most stable. However, the energy differences are small and AM1 energy minimizations were performed without any distance constraints. Nevertheless, the comparison of the corresponding geometries is more encouraging. The small RMS value between AM1 and DISCOVER optimized structures (Figure 4a, 4b) suggests that the DISCOVER predicted conformations also

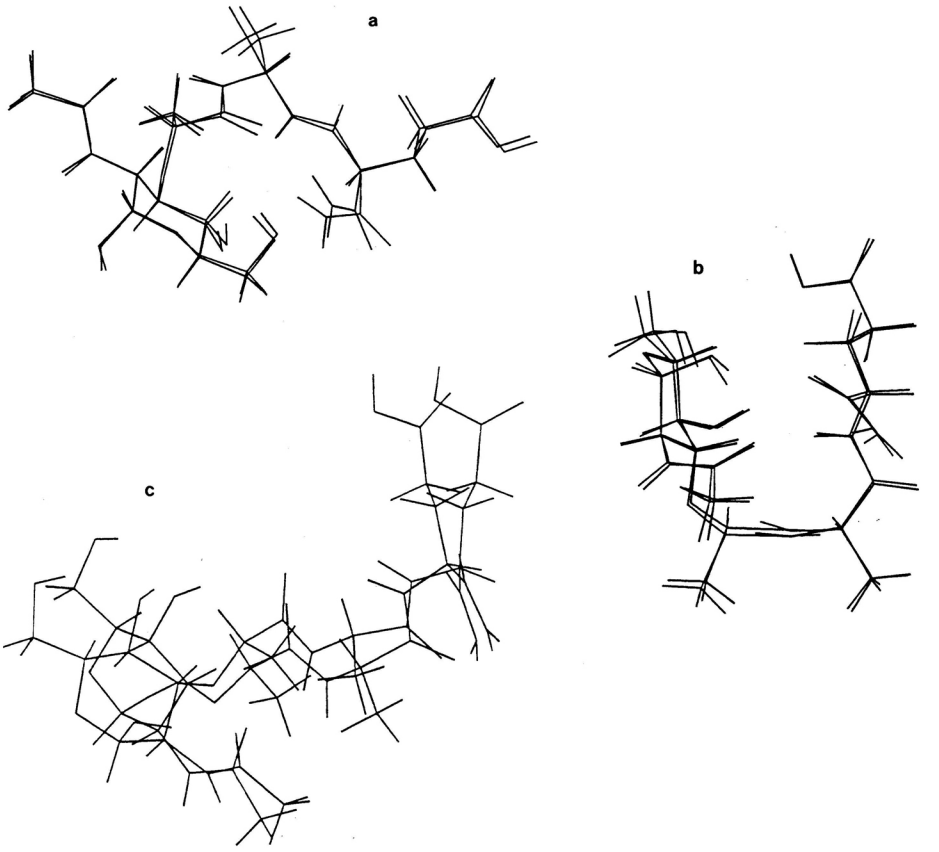

Figure 4. **a, b**: Superpositions of the two lowest DISCOVER energy minimized structures and the corresponding structures as results of AM1 energy minimization. **c**: Superposition of the extended DISCOVER structure with AM1.
Note that **a, b** DISCOVER structures are the result of the restrained energy minimization and **c** DISCOVER and all the AM1 structures result from full energy minimizations. RMS values for DISCOVER and AM1 structures are 0.206, 0.161 and 0.976 Å for the conformations **a, b** and **c**, respectively.

correspond to the AM1 predicted conformations, although in the latter calculations no distance constraints were introduced.

It might be expected that, according to the experimentally determined distance constraints, the existence of the H-bond 2 stabilizing the second β II turn is doubtful and significant fluctuations in the isoglutamine side chain orientation take place in the DMSO solution. The experimentally determined quantities in such a dynamic system are no more simply related to the population of various conformations. The observed NOE falls off as r^{-6} and, therefore, determination of the number of conformations contributing to the measured value and of their corresponding Boltzmann factors is not a trivial matter.

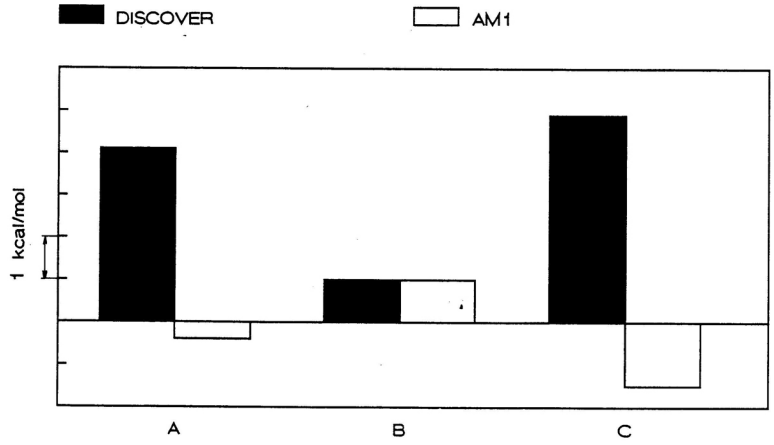

Figure 5. Comparison of DISCOVER and AM1 energies for the structures in Figure 4.

Any of the low energy conformations might adapt to the receptor's requirement. Considerations of the influence of substitutional effects on conformation and activity should narrow the choice of eligible conformations. The reasoning of Fermandjin *et al.*[8] on the changed activity spectrum of murabutide (Mur-*N*-Ac-L-Ala-D-Gln-*On*Bu), which retains only H-bond 1, is an example of this sort of approach to the problem of bioactive conformation. In this vein, we are extending the theoretical modeling to several constitutionally different peptides of similar activity as well as to inactive ones with a similar structure.

The results of the present computational approach and of the NMR conformational analysis[1] are not in full agreement. The reason may lie in the deficiencies of the adopted force field or, more likely, in the lack of the explicit consideration of medium effects. In any case, the theoretical prediction of the existence of families of conformations with very similar energies leaves open the question of the conformation that best fits the putative receptor.

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IZVLEČEK

Konformacija imunomodulatorja muramil peptida v raztopini. Študija z molekularno dinamiko, omejeno s podatki jedrske magnetne resonance in s semiempirično MO metodo

Viljem Harb, Janez Mavri, Jurkica Kidrič i Dušan Hadži

Konformacijo *N*-acetil-muramil-L-alanil-izoglutamina (MAG) smo analizirali z molekularno dinamiko. Začetne geometrije smo določili z naključnim spreminjanjem dihedralnih kotov. Obsežen konformacijski prostor smo skrčili z vključitvijo eksperimentalnih podatkov o NOE povezavah in intramolekularnih vodikovih vezeh v potencialno funkcijo. Energijske razlike med sedmimi strukturami z najnižjo celotno energijo so zelo majhne. Energijski vrstni red struktur minimiziranih s semiempirično MO metodo AM1 ni enak tistemu, ki ga napove molekularna mehanika, vendar so razlike majhne. Konformacija v obliki črke S, napovedana s pomočjo eksperimentalnih JMR podatkov, je energijsko možna.