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EFFECT OF SUBSTITUTION OF D-ALANINE FOR L-ALANINE ON ACTIVITY AND CONFORMATION OF AN ENCEPHALITOGENIC PEPTIDE

G. Khanarian, S.A. Margetson, W.J. Moore and S.J. Pasaribu
School of Chemistry, University of Sydney, N.S.W. 2006 Australia

F. C. Westall
Salk Institute, P.O. Box 1809, San Diego, CA 92112

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SUMMARY : Synthetic peptides Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys and its D-Ala analog were tested for induction of experimental allergic encephalomyelitis (EAE) in guinea pig. The L-Ala peptide was highly active at 0.5 µg dosage and the D-Ala peptide was inactive even at 10 µg dosage. NMR spectra indicated backbone conformational differences between the two isomers. Energy calculations delineate conformations that are high in energy for the D-form and low for the L-form. A conformation for the physiologically active peptide is suggested that is in accord with both clinical and physical data.

Experimental allergic encephalomyelitis (EAE) is an inflammatory demyelinating disease of the central nervous system caused by a cell-mediated immune response to myelin basic protein (MBP) or peptides derived from it. The encephalitogenic determinant in guinea pig has been localized in residues 114 to 122 of MBP (bovine sequence) Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys (1). The marked loss in activity caused by changes in the amino-acid residues at Gly^4-Ala^5(2,3) suggests that a bend in the peptide chain in this region and steric hindrances by the methyl group at Ala^5 may be important in fixing the encephalitogenic conformation.

EXPERIMENTAL : The D-Ala^5 peptide was synthesized by the same method as described for the L-Ala^5 peptide (4), except that t-boc-D-alanine (Bachem) was used as a starting material ($[\alpha]_D^{22}$ = +25.20, t-boc-L-alanine $[\alpha]_D^{22}$ = -25.15, both 0.20 M in acetic acid.) The purity of the peptides was checked by elemental and amino-acid analyses as well as by extensive NMR spectra at each stage of the synthesis.

Activities of the two peptides in causation of EAE in guinea pigs were tested by the standard protocols developed at the Salk Institute(5). Thirty-two female albino guinea pigs were injected in each hind footpad with variable doses of either the L-Ala or the D-Ala peptide. Just prior to injection, the antigen was dissolved in normal saline and emulsified with Freund's complete adjuvant in the ratio 1:3. Commercial Difco complete adjuvant with 2 parts mineral oil containing Mycobacterium tuberculosis with H37Ra (Difco No. 3113-605 Bacto adjuvant) and one part of Aquaphor was used. After injection, the animals were observed for 28 days. Clinical

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TABLE 1. Biological Tests of Encephalitogenic Peptides in Guinea Pigs.

Peptide	Dose µg	Clinical	Day of Onset	Histological
FSWG(L-A)EGQK	0.50	2/4	11-15	3/4
	1.00	3/4	11-13	4/4
	5.00	3/4	13-14	4/4
	10.00	2/3	13-14	3/3
FSWG(D-A)EGQK	0.50	0/4	-	-
	1.00	0/4	-	-
	5.00	0/4	-	-
	10.00	0/4	-	-

signs were recorded and histological examinations made of brains of the animals at the end of the experiment. Clinical signs of the disease included incontinence, diarrhoea, and hind-leg paralysis.

The ^{13}C NMR spectra of the D-Ala and L-Ala peptides were recorded at 22.625 MHz on a Bruker HX-90 spectrometer. Solutions 0.10 M in D_2O were studied from pD 1.9 to 10.4 at 298 K. The internal standard was p-dioxane with an assigned chemical shift δ = 67.86 ppm downfield from external tetramethylsilane. Assignments were made by comparisons with data on component and model (6) peptides, variations of δ with pD, and single proton decoupling (7). An average of 16 spectra over the pD range were used for comparison of the two peptides.

The 270 MHz ^1H spectra were recorded on a Bruker HX-270 spectrometer (National N.M.R. Centre, Canberra, A.C.T.). Solutions 0.02 M in 90% H_2O/10% D_2O were studied from pH 2.0 to 8.0 at 298 K. Assignments were made by comparisons with published chemical shifts, integrated peak intensities, multiplet structures, pH dependence of δ, and homonuclear decoupling. Chemical shifts are reported relative to internal sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate.

Theoretical energy calculations were made in accord with the methods of Momany et al.(8). The computer program was QCPE 286 (Empirical conformation energy program for peptides). The total energy was minimized by an algorithm due to Powell (9).

RESULTS AND DISCUSSION : The results of the clinical tests are in Table 1. In brief, the L-peptide was active but the D-peptide was inactive in all doses.

In the ^{13}C-NMR spectra, the effect of the D-Ala for L-Ala substitution is seen primarily in backbone carbons. The pD dependence of chemical shift δ in some cases differs in the two peptides. The effective pK values of these titration shifts identified the ionizing groups cited in Table 2. In the case of the L-peptide the group whose pK corresponds to

TABLE 2. Differences in Chemical Shifts between L-Ala and D-Ala Peptides (L-D) and titratable groups that govern the pH dependence of shifts.

pH	3.0	4.0	5.0	6.0	7.0	Group
W^3-α	0.3	0.3	0.2	0.1	0.1	E*
G^4-C^1	0.2	0.2	0.2	0.3	0.3	F(L)
A^5-α	0.0	0.0	0.0	0.1	0.1	E+K-COOH
A^5-β	0.0	-0.1	-0.1	-0.1	-0.1	K-NH_3^+(L)
E^6-α	0.1	0.1	0.1	0.1	0.2	E ; K-NH_3^+(L)
E^6-β	-0.1	-0.1	-0.1	-0.2	-0.2	E
E^6-γ	0.0	0.2	0.1	-0.1	-0.2	E ; K-COOH(L) ; F-NH_3^+(L)

* Δ_L = -0.2, pK 4.5; Δ_D = +0.1, pK 4.6

the titration shift of a backbone carbon is sometimes quite far removed along the peptide chain, but the D-peptide titration shifts are usually associated with groups nearby in the chain.

In the proton spectra the most significant indication of conformation is provided by the dependence of δ on pH for the two nonequivalent amide protons of the glutamine sidechain. One of these (syn) remained constant at 6.86 ppm from pH 2.0 to 8.0, whereas the other (anti) moved from 7.50 ppm at pH2.0 to 7.58 ppm at pH 8.0, following a titration curve corresponding to the pK = 4.5 of the Glu sidechain. This effect was accentuated in 10% aqueous dimethylsulfoxide solution where the shift was from 7.50 to 7.64 ppm. This result suggests that there is a hydrogen bond between the amide of Gln^8 and the carboxyl of Glu^6 . Such a structure in the physiologically active conformation of the peptide would explain the requirement of these two residues at positions 6 and 8 for high encephalitogenic activity (10).

A linear nonapeptide in aqueous solution can exist in many different conformations. At 298 K conformations with an energy more than 10 kJ above the minimum have negligible concentrations. If the rate of interchange between low-energy conformations is fast on the NMR time scale (>10^9Hz) the NMR spectrum corresponds to a

weighted average of the accessible conformations. In a physiological test, however, a cell receptor may respond to only a narrowly defined range of conformations of the effector molecule. The difference in activity between the D-Ala and the L-Ala forms of the peptide antigen may be due either to a difference in the accessibility of the methyl group at Ala^5 or to a change in conformation of the entire peptide caused by the D- for L- substitution. The clinical data on -Gly^4Gly^5- and -Ala^4-Ala^5- peptides favours the latter explanation. The NMR data indicate that the conformation of the L-Ala peptide differs from that of the D-Ala peptide. The pattern of "long range" titration shifts in the L-Ala but not the D-Ala peptide would be consistent with a bend in the middle of the L-Ala chain.

Theoretical calculations of the energies of peptides have been useful in conformational studies (11,12). The energies do not explicitly include effects of neighboring solvent molecules, but the calculations provide a systematic way of eliminating structures that are most unfavorable in their nonbonded interactions. The clinical differences in activity of L-Ala and D-Ala peptides and the NMR data narrowed the range of conformations to be considered. It is difficult to apply these methods to a complete nonapeptide, and various segments were examined separately and then put together to yield a final conformation.

The computed conformational energy of the nonapeptide was investigated in three parts: Phe-Ser-Trp, Trp-Gly-Ala-Glu, and Glu-Gly-Gln-Lys. Because of the large conformational space available to these peptides, it was necessary to choose reasonable starting positions for the minimization of the energy. Zimmerman et al. have tabulated extensive lists of low-energy conformations of the N-acetyl-N' methylamides of individual amino acids (13) and many

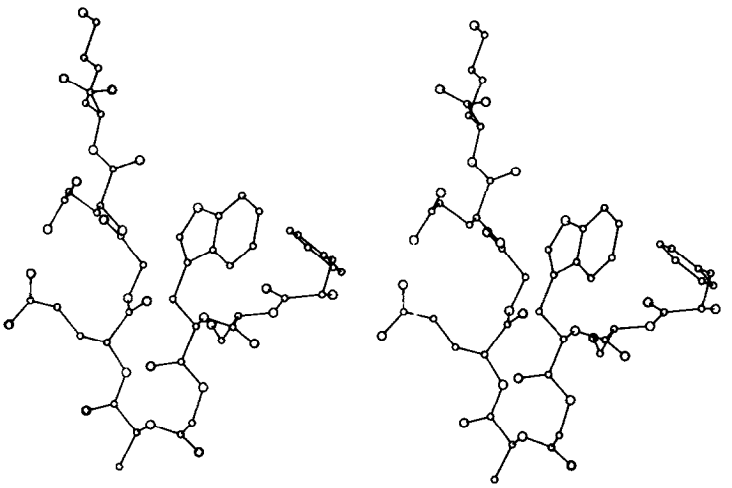

FIG. 1. Stereoscopic drawing of possible encephalitogenic conformation of the nonapeptide Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys (FSWGAEGQK). The C-terminal lysine is at the top of diagram.

dipeptides (14). The various bend structures given by Lewis et al. (15) were also used as starting positions for the central Gly-Ala segments.

The lowest energy of Trp-Gly-Ala-Glu was -50 kJ/mol and there were 8 other conformations within 8 kJ/mol of the minimum, and of these, 6 were bend conformations. For the tripeptide Phe-Ser-Trp, the lowest energy was -35 kJ/mol and there were 11 other conformations within 8 kJ/mol of the minimum. For the tetrapeptide Glu-Gly-Gln-Lys, the starting angles of the Glu and Gln residues were such that there was a hydrogen bond between one of the amide hydrogens of Gln and the carboxyl of Glu, as indicated by the NMR studies. The lowest energy of this C-terminal tetrapeptide was -93 kJ/mol, and there were 5 conformations within 8 kJ/mol of the minimum.

When the fragments were brought together to form the nonapeptide, the following conformations were used as starting positions:

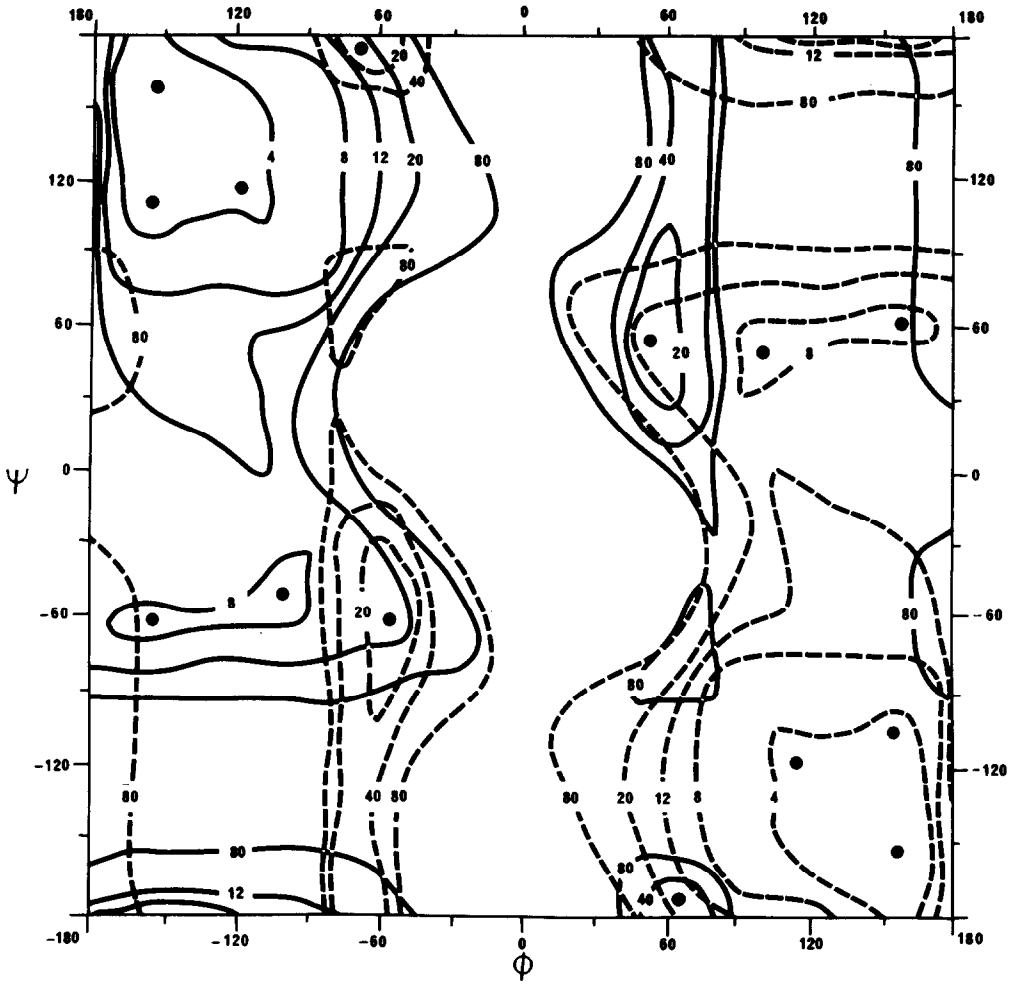

FIG. 2. Conformational energy maps of N-acetyl-N'-methylamides of L-alanine (solid lines) and D-alanine (dashed lines). The solid circles indicate minima in energy.

the four lowest-energy conformations of Phe-Ser-Trp and the lowest-energy conformation of Trp-Gly-Ala-Glu were combined with the five lowest-energy conformations of Glu-Gly-Gln-Lys. Thus there were 20 starting positions for the nonapeptide. The energy was minimized with respect to all dihedral angles and the lowest-energy conformation, at -72 kJ/mol, is shown in Fig. 1.

The active conformation is believed to be one of low-energy for the L-Ala peptide and high energy for the D-Ala peptide. The conformation space for the residue Ala^5 is shown in Fig. 2 for the L and D peptides. We eliminate regions in which both forms are low in energy, as well as regions in which the D-form is much lower in energy than the L-form. We are then left with the region ϕ = -150 to -80, ψ = 60 to 150. The calculated minimum-energy structure of the L-peptide lies in this region and the calculated energy of the D-nonapeptide in this conformation lies 30 kJ/mol higher than that of the L.

We also examined a conformation similar to the one suggested by Carnegie and Smythies (16), in which the bend occurs at Ala-Glu instead of Gly-Ala. Although its minimum energy was 20 kJ above that with the Gly-Ala bend, this conformation is still under consideration.

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