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Conformational studies of nonapeptide using NMR

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The present study describes the study of conformation of nonapeptide Tyr-Met-Asp-Gly-Thr-Met-Ser-Gln-Val (YMDGTMSQV), capable of being recognized by melanoma specific T-cells, corresponds to residue 369 to 377 of tyrosenase gene. Secondary structures have been obtained by ^{1}H NMR and molecular dynamic simulation using CSI values, 2D COSY, NOESY and ROESY, at 500 and 600 MHz in solvent DMSO-d_6. On this basis, nonapeptide shows a γ bend and non-ideal type 1 β-turn.

Keywords: NMR data, nona peptide, melanoma, epitope, γ bend, non-ideal β-turn

There is growing interest in the study of conformation and folding pattern of small and midsized (<30 residues) polypeptides because sequence of epitopic peptide and peptide hormone fall in this range. Besides this small peptides with more ordered conformation and structural peculiarities represent a model piece for various physico-chemical studies, e.g. helix Geometry. It has been reported that the peptide-binding site of human Class-I histo compatibility molecule recognizes epitopic peptides consisting of 8-11 amino acid residues[1]. Of several such peptides only few are recognized by human melanoma specific cytotoxic T-lymphocytes.

Conformational study of small peptide deserves recognition in view of the (i) significance of melanoma in the field of cancer immunotherapy, (ii) it triggers immune response in several patients.

An attempt has been made to investigate one such epitopic peptide, a nonamer Tyr-Met-Asp-Gly-Thr-Met-Ser-Gln-Val (YMDGTMSQV) which has been shown to have high affinity for melanoma specific CTL cell lines from five different patients. Residue 369-377 of tyrosinase gene code for the peptide formed as a result of post translational modification[2]. Clinical trials of this nonapeptide based vaccine is in progress and have shown favourable response[3-5]. Studies are also in progress to understand the processing and presentation of the membrane protein using tyrosinase gene as a model[6].

To understand on structural ground the mechanism of interaction of the nonapeptide with T-cell receptor, we carried out NMR studies and MD simulation which shows that nonapeptide adopts ordered conformation in DMSO. It has been found to be associated with β turn and γ bend. In a similar study of one such epitopic fragment 579-601 in the gp41 trans membrane domain of HIV-1 by co-authors Prashant *et al*. using NMR and MD simulation, have shown that it adopts a β pleated sheet in DMSO[7].

NMR Experiments

The nonapeptide, tyrosinase gene fragment, was purchased from Celtek Bioscience, LLC Nashville, TN 37210, U.S.A. About 5 mg of nonapeptide was dissolved in 0.6 mL DMSO-d_6 in a nitrogen atmosphere to minimize the moisture content. The NMR experiments were carried out on 500 MHz AMX Bruker NMR spectrometer and Varian units plus 600 MHz FT NMR spectrometer.

Spin system assignment has been carried out using double quantum filtered correlated spectroscopy (DQF-COSY)[8] and total correlation spectroscopy (TOCSY)[9]experiments. All TOCSY spectra were recorded with 512 experiments (16 and 32 scans each, 1.5 and 1.0s relaxation delay for 500 and 600 MHz respectively) and an MLEV 17 mixing scheme of 80 ms pulse was used. Nuclear overhauser enhancement

spectroscopy (NOESY)[10] and rotating frame nuclear overhauser effect spectroscopy (ROESY)[11] spectra were used for sequential assignment. The NOESY spectrum was recorded with mixing time ranging from 50 to 500 ms, 1.5s relaxation delay, 16 transients and 512 experiments for 600 MHz and 1.0s relaxation delay, 32 transients and 512 experiments for 500 MHz. All NOESY spectra were acquired in the hypercomplex (STATES) method[12]. The data was apodised with a sine bell window function and zerofilled to a matrix of size 2K of size 2K ×1 K data points to fourier transformation. The spectra were calibrated relative to resonance peak of DMSO-d_6 which was 2.5 ppm downfield relative to tetramethyl silane (TMS).

NOE intensities and refinement of interproton distances

Interproton distances were calculated from NOESY cross peak intensities using the following relation[13]

$$r_{ij} = r_{k1} (I_{k1}/I_{iij})^{1/6}$$

where r_{ij} and I_{ij} refer to the distance and the integrated NOE volume between two portions of interest, and r_{k1} and I_{k1} refer to the same quantities for a pair of reference protons. The βH_1 and βH_2 protons of tyrosine are nondegenerate and their internuclear distance is invariant with the peptide conformation. Therefore, this internuclear distance 1.7 A has been taken as a reference distance.

MD simulations

MD Simulations was carried out using NOEs distance restraints for nonapeptide in DMSO-d_6. Simulations were done on a silicon graphics Indigo work station with molecular modeling software Insight II V 2.3 and Discover V2.9 from MSII, USA. The energy of the system was evaluated with the consistent valence force field[14]. In this, the bond stretching was described by a simple harmonic function and cross terms were not described for the system. The dielectric constant was set to 1.0.

Prior to MD Simulations, the system was energy minimized by the method of SD (1000 steps) followed by CG (2000 steps). An upper and lower bound to the distance was set and both lower and upper bond force constants fixed at 30 kCal/mole A°. The maximum force permitted to satisfy the constraints was set as 1000 kCal/mole. Since steroeospecific assignments for β, γ protons were not possible, for these individual group of atoms, a centroid was specified and distance restraint defined to this centroid. MD Simulation were started with a 1.0 ps equilibriation at 300K, which was then followed by the data collection period of 25 ps. A frame for this 25 ps trajectory were captured every 1.0 ps. Each frame was energy minimized to a gradient of 0.01 Kcal/mole/Å. The saved structure were energy minimised by SD (1000steps) and CG (2000 steps)

Resonance assignments

The one dimensional (ID) spectrum of nonapeptide was recorced in DMSO-d_6. All the NH peaks except terminal Tyr NH are well resolved and observed between 7.6-8.6 ppm. The first step in resonance assignment is to classify the pattern of TOCSY cross peaks according to spin system of an amino acid with reference to empirically known chemical shift positions[15]. **Figure 1** shows a portion of the TOCSY spectrum of nonapeptide in DMSO-d_6. The unique spin system of Gly, Val and Thr helps to identify them unambiguously. The AMX spin system group i.e. Tyr-1 Asp-3 and Ser-7 were also identified. The resonances arising from the backbone NH-CαH protons of the two Met residues overlap at room temperature therefore the AM (PT)X group of Met-2 and Met-6 was identified with γ protons present at lower chemical shift in the ROESY Spectrum. The AM (PT)X spin system group of Gln-8 was independently identified from the strong γCH-δNH cross peak in the ROESY spectrum.

The second step in assignment, the sequence specific assignments were obtained using NOSEY spectrum where the cross peak of NH CαH of residue i was linked to that of residue i + 1 sequentially through the NOE peaks along the main chain of the peptide (**Figure 2**). Of the nine amino acid residues only Tyr NH-CαH do not show coupling correlations because of its terminal position.

The break in sequential assignment for nonapeptide at C-2 and C-6 due to overlapping peaks Ser-7 (NH) - Met-6 (CαH) and AsP-3 (NH) - Met-2 (CαH) was joined by the observation of NOEs between NH protons of Ser-7 - Met-6 and Asp-3-Met-2-C-βH. Other important NOEs which supports the sequential assignments are cross peaks between Gly-4 NH and Asp-3 NH and between Val-9 NH and Gln-8 NH.

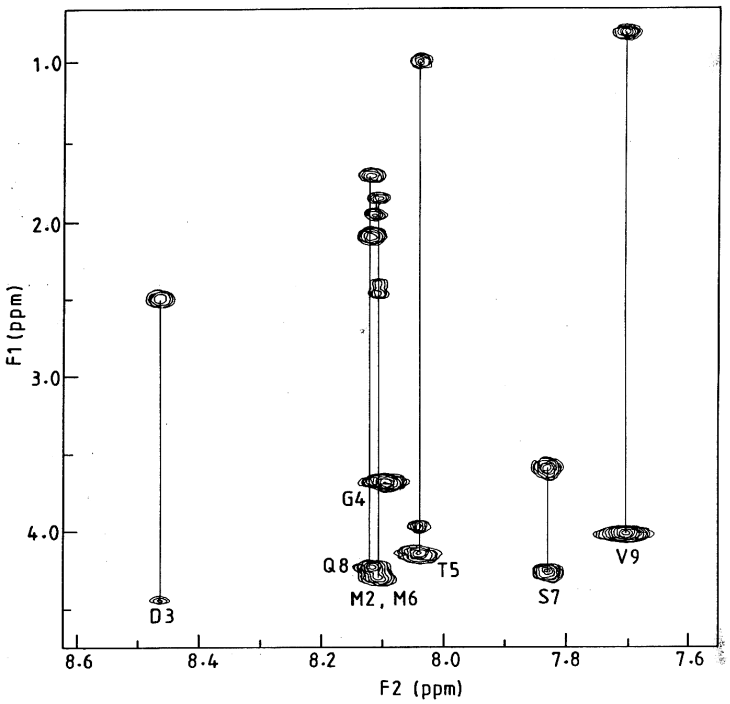


Figure 1 — 600 MHz TOCSY spectrum of nonapeptide in DMSO-d_6 NH-CαH cross peaks are labled with single letter code for amino acids

Results and Discussions

The conformation of nonapeptide was derived from chemical shift index (CSI) values and observation of nuclear overhauser effects.

Chemical Shift Index

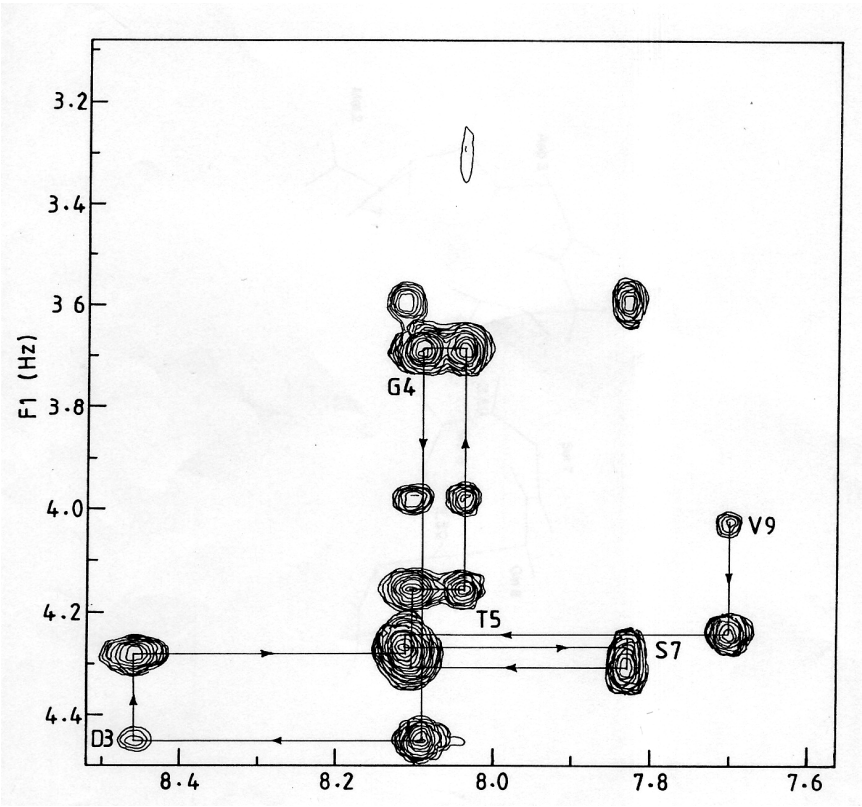
The CSI of a proton is defined as the difference between the measured width and the chemical shift for proton in a random coil structure[16]. A positive deviation from the random coil chemical shift is an indicative of a β-sheet while negative deviation suggests helical or turn like structure. **Table I** shows CSI values for the NH and C-αH protons of nonapeptide. The negative CSI values for residues except Asp suggest the possibility of a turn like structure in the molecule between residue **4-9**.

Temperature Coefficients

Temperature coefficients of NH chemical shifts have been measured from 1D spectra recorded in the temperature range of 298-323k. These values fall between 2.2 and 5.1 ppb/k except for aspartate[17,18] and are in general lower than those expected for solvent exposed NH protons. The temperature coefficients can be grouped in three ranges. Below 3.00 ppb/K are strongly indicative of intramolecular H-bonds. Those in the range 3.0 - 5.0 ppb/K the intermediate range are suggestive of NH protons which are in dynamic equilibrium between intramolecularly hydrogen bonded form and structures with those protons easily accessible to solvent. Above 5.0 ppb/k the NH protons are freely proposed to solvent. In the present case the NH protons of Ser-7 and Val-9 have temperature coefficients below 3.0. which clearly indicates involvement of these residues in H-bonding. Temperature coefficient values of rest of the amino acids residues are in the intermediate range <5.0 ppb/K indicating a dynamic equilibrium between a H-bonded conformation and a non H-bonded form.

NOE Data

A large no. of both inter and intraresidue NOEs are seen for nonapeptide. A summary of the most of the observed NOEs is given in **Figure 3**. All sequential NHi - NHi + 1 and C-αHi - CαHi+ 1, CαH - NHi+ 1 NOEs have been observed. A significant number of long range NOE T5NH - S7 NH have also been observed which suggest the possibility of a definite structure associated with residues 4 - 9 of the



V_9 Val NH-α
S_7 Ser NH-Met-α
T_5 Thr NH-α
G_4 Gly-α-Thr NH
D_3 Asp NH-α

Figure 2 — NH-CαH region of NOESY spectrum in DMSO-d_6 at 298k recorded with mixing time of 300 ms

nonapeptide. Presence of C-αHi - C-αHi+1 NOEs, e.g., M-2 (CαH) - D-3 (CαH), T-5 (CαH) - M-6 (CαH), G-4 (CαH) - T-5 (CαH) further supports the possibility of a definite structure at the C-terminal end.

Molecular Dynamics (MD) Simulations

The backbone torsion angle (ϕ,ψ) averaged over the entire trajectory for nonapeptide is presented in **Table II**. The root mean square deviation (rmsd) of the ϕ, ψ angle of various structures of nonapeptide generated by simulation is zero which indicates a very good homogeneity of the simulated structures.

No violation in the imposed distance restraints was observed in this structure. The secondary structures that emerges for nonapeptide from above studies show a β turn around Met-6 Ser-7 Gln-8 Val-9. Although the ϕ,ψ angle do not exactly match those in the standard type turns [15], the H-bonding patterns, a signature of the β-turn was clearly observed Le., the Co of Met-6 is H-bonded to NH of Val-9. Value of ϕ, ψ angles (for Ser - 43.71 - 41.66 and Gln - 120.44, 58.65) combine with hydrogen bonding pattern bring the turn closer to type 1 β-turn[19] Another important observation is the presence of a bend formed at Met-6 without giving any restraints in MD simulation, thereby satisfying the low temperature coefficient value of Ser-7[20].

Conclusion

Structural studies of epitopic peptides have shown that monomers decamers are the most favoured candidate for TCR. The conformational study of nonapeptide YMDGTMSQV which corresponds to residue 369-377 of tyrosinase gene has been reported.

Table I — CSI values of NH and CαH proton of peptide in DMSO-d_6

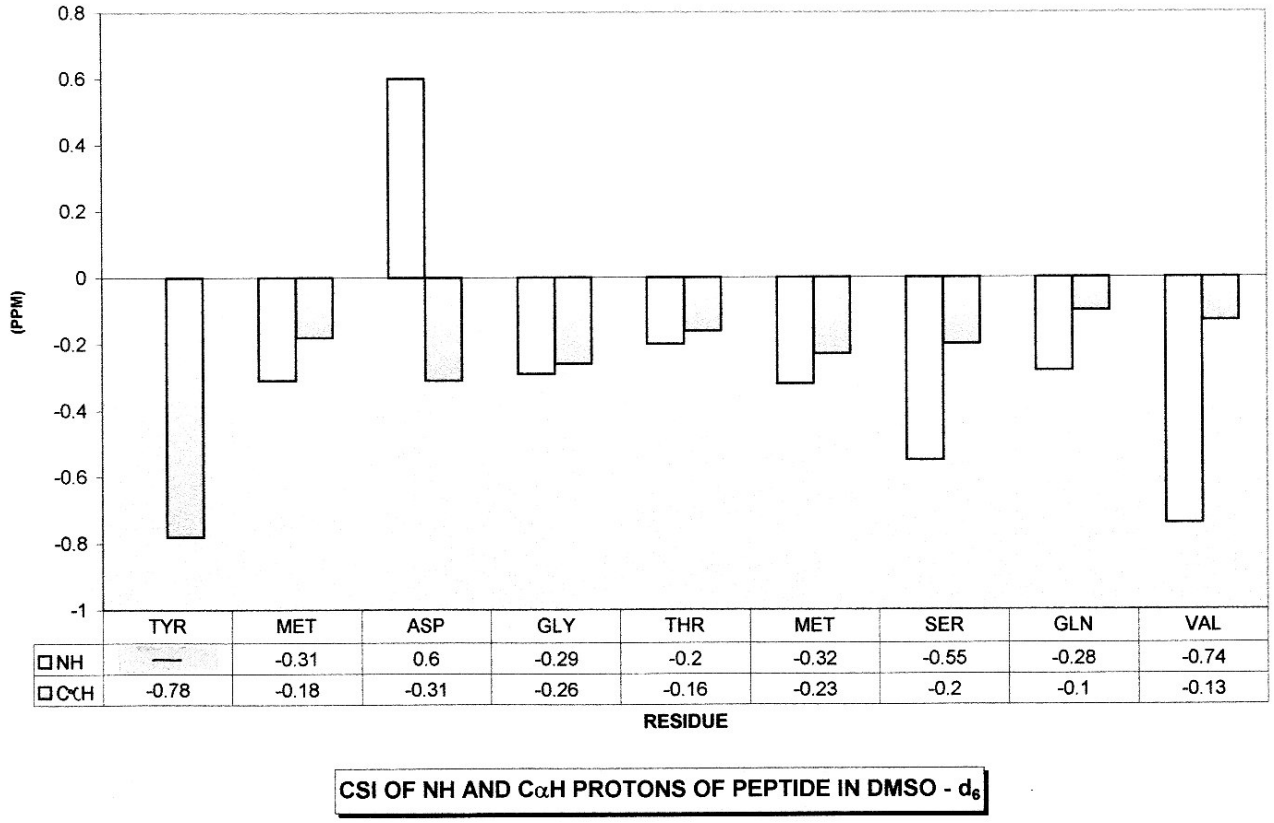


Table II — Backbone Torsion ang1es (ϕ,ψ) for nonapeptide averaged over the entire MD Trajectorv in the MD Simulation

a, b Dihedral Angles Residue	Simulation	
	ϕ	ψ
1Tyr	-	112.29
2Met	-150.00	71.17
3Asp	-158.00	78.74
4Gly	146.09	105.52
5Thr	-91.46	-143.91
6Met	-64.77	-23.45
7Ser	-43.71	-41.66
8Glv	-120.44	58.64
9Val	-131.75	176.12

[a]In degrees
[b]Value in parenthesis is the rms deviation

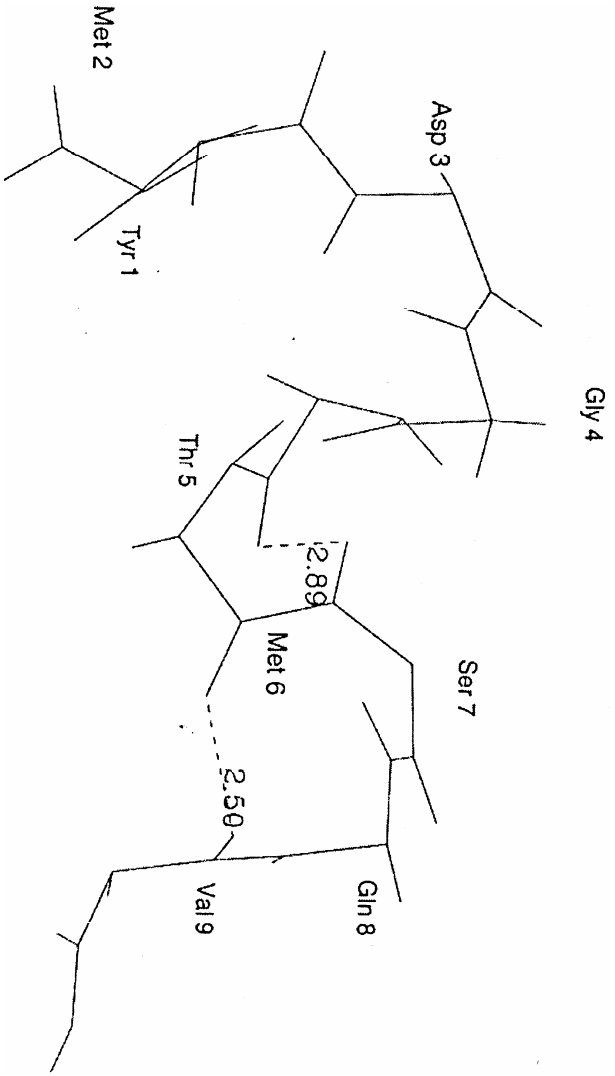


Figure 3 — Summary of the important NOE's for the nonapeptide in DMSO-d_6

Our experimental study indicates that nonapeptide in DMSO is associated with a β-turn around Met - Ser - Gin - Val. Another notably feature observed is a Y bend at Met-6. Clues regarding the secondary structure can be obtained from CSI values, NOE data and MD simulation studies. The negative CSI values for residues except that Asp suggests the possibility of a turn like structure in the molecule between residues **4-9**.

NH protons of Ser-7 and Val-9 have temperature Co-efficient below 3.0 which clearly indicates involvement of these residues in H-bonding. The results of molecular modelling are in agreement with these data. A H-bond form Met (Co) to Val (NH) is clearly observed. All sequential CαH -NH, NHi-NH+1- NOE's with a strong NOE between T_5NH-S_7NH is also observed. On the basis of ϕ and ψ angles for Ser-secondary structure seems to be a non-ideal type 1 β-turn.

The result has been found to corroborate with the crystal structure of Major Histocompatibility (MHC) Class-I molecule and HLA complex with nonameric/decameric peptide deposited at protein data bank. It should be noted that the presence of an intra peptide H - bond and a sharp bend in the peptide to accommodate it in the groove has also been shown by Hulsmeyer *et al.* in the study of crystal structure of one such peptide complex i.e. Human Leukocyte Antigen (HLA - A 0201), Melanoma Antigen Gene (MAGE –A_4), decameric peptide, HLA complex[21].

Study of the above complex has shown that amino acid valine, present at C-terminal residue, plays an important role in nestling the peptide in one of the grooves of MHC Class-I molecule. Since valine is present as C-terminal residue in nonopeptide it probably plays a similar role here also. Fasman *et al.* have noticed similar structural deviation in case of β-turns and their existence in equilibrium with a ϒo bend[22].

In view of the above reports we conclude that our study could throw some light on conformational peculiarities associated with epitopic peptide which might also contribute to the binding of T-Cell receptor to it.

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