Conformational Analysis of a Glycosylated Human Myelin Oligodendrocyte Glycoprotein Peptide Epitope Able To Detect Antibody Response in Multiple Sclerosis

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Myelin oligodendrocyte glycoprotein (MOG), a minor myelin component, is an important central nervous system specific target autoantigen for primary demyelination in autoimmune diseases such as multiple sclerosis (MS). The native structure of MOG presents a glycosylation site at position 31 (Asn[31]). It has been recently described that glycosylation of a MOG peptide epitope improved the detection of specific autoantibodies in sera of MS patients. The solution conformational behavior of two MOG derived peptides—hMOG(30–50) (**1**) and the glycosylated analogue [Asn[31](N-β-Glc)]hMOG(30–50) (**2**)—were investigated through NMR analysis in a water/HFA solution. Conformational studies revealed that peptides **1** and **2** adopted similar conformations in this environment. In particular, they showed strong propensity to assume a well-defined amphipatic structure encompassing residues 41–48. The N-terminal region resulted to be almost completely unstructured for both peptides. The presence in **1** of a low populated Asx-turn conformation characteristic of the Asn-Xaa-Thr glycosylation sites was the only conformational difference between peptides **1** and **2**. Thus, the specific antibody recognition of peptide **2** is most likely driven by direct interactions of the antibody binding site with the Asn-linked sugar moiety.

Introduction

Multiple sclerosis (MS) is a central nervous system inflammatory demyelinating disease whose pathogenesis is very likely mediated by an autoimmune response directed to structures of the myelin sheath. However, despite a large effort by numerous research groups, the target molecules of this autoimmune attack are still unknown.[1] In recent years, interest has focused on minor myelin antigens among which myelin oligodendrocyte glycoprotein (MOG) is an interesting candidate autoantigen. MOG is specifically expressed in the central nervous system (CNS), and it is located on the outer surface of the myelin sheath and therefore exposed for antibody attack.[2] Anti-MOG antibodies are known to play a key role in the development of demyelination in a number of in vivo and in vitro experimental systems. In MS a predominant T cell response to MOG has been reported, while little is known about antibody reactivity to MOG.[3] However, thus far all the studies investigating B cell autoreactivity to MOG have used recombinant protein or synthetic peptides which do not contain the glycosyl moieties of this glycoprotein. It is possible that the difficulties in identifying antibodies directed to MOG peptides in humans are related to the fact that these antibodies recognize a glycopeptide structure. MOG has a glycosylation site at Asn[31] but the molecular features of the glycosyl moieties as well as the conformational structure of the native glycoprotein are largely unknown.[4] On the other hand, post-translational modifications of proteins, such as glycosylation, may dramatically affect their antigenic properties.[5–6]

We have previously shown that the conjugation with a very simple glucosyl moiety of a MOG peptide encompassing the immunodominant region and containing the native glycosylation site resulted in improved detection of specific autoantibodies in humans. In addition, autoantibodies detected by the MOG glycopeptide were found in a relevant number of MS patients, and their titres were correlated with disease activity.[7] Thus, the peptide [Asn[31](N-β-Glc)]hMOG(30–50) (**2**, sequence: K[30]N(β-D-Glc)ATGMEVGWYRPPFSRVVHL[50]) is a synthetic antigen able to detect pathogenic demyelinating autoantibodies in MS patients and, most importantly, it can be used as a template for the design of a new generation of drugs capable of specifically block circulating autoantibodies in patients affected by MS. In view of this goal, we describe here a conformational analysis of this peptide, in comparison with the unglycosylated counterpart, hMOG(30–50) (**1**).

Results and Discussion

NMR Analysis. The antibody binding activity displayed by the glycopeptide **2** can be due to either direct interactions between the carbohydrate moiety and the antibody binding site, or an influence of the carbohydrate moiety on the conformations which can be adopted by the peptide portion. In an attempt to investigate these alternatives we have studied the conformational differences between **1** and **2** using NMR spectroscopy and molecular modeling.

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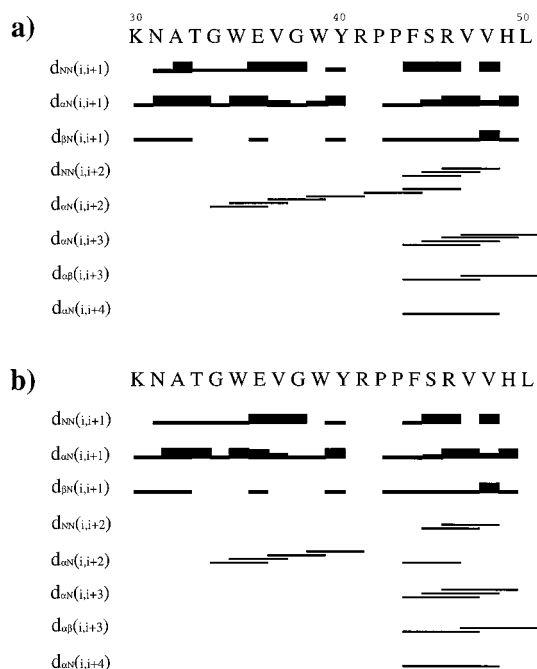[#] University of Salerno.
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a)

b)

Figure 1. Schematic diagrams showing the NOE connectivities observed in the NOESY spectra of peptides **1** (a) and **2** (b). Thickness of the bars is related to the NOE intensities.

Table 1. Amide Proton Temperature Coefficients ($-\Delta\delta/\Delta T$) of Peptides **1** and **2**[a]

	1	**2**		**1**	**2**
Asn 31	3.64	3.72	Tyr 40	2.52	2.82
Ala 32	6.26	6.26	Arg 41	2.20	2.60
Thr 33	4.62	6.14	Phe 44	4.90	5.22
Gly 34	5.08	4.80	Ser 45	3.34	4.06
Trp 35	2.92	2.36	Arg 46	1.06	1.46
Glu 36	3.34	2.66	Val 47	1.56	1.78
Val 37	4.90	5.42	Val 48	4.88	4.90
Gly 38	4.70	4.02	His 49	2.10	2.22
Trp 39	3.14	3.84	Leu 50	3.68	3.82

[a] The temperature coefficients ($-\Delta\delta/\Delta T$) of the amide protons are listed in ppb/K. They are the results of a linear regression analysis of the chemical shifts measured in the range 290–310 K and accurate to within ±0.4 ppb/K.

The usual high conformational freedom of linear short peptides in water solution leads to inextricable mixtures of isoenergetic conformers. The use of a solvent system having suitable viscosity and polarity properties allows the most energetically stable conformers to prevail, thus making the NMR spectra more interpretable.[8] Therefore we recorded spectra in a water/HFA (50:50, v/v) mixture. HFA (hexafluoroacetone trihydrate), which is a typical structure stabilizing cosolvent, acts by favoring the intramolecular hydrogen bonds and consequently folded conformations.[9]

Almost complete ^{1}H chemical shift assignments of peptides **1** and **2** were achieved (Supporting Information). Qualitative evaluation of NOE connectivities in the NOESY spectra (Figure 1), secondary shift of the C$^\alpha$H (Supporting Information),[10] and amide proton variable temperature coefficients (Table 1) were suggestive of a helical conformation encompassing residues 43–48 while the same data indicated a less defined nascent helix[11] along residues 32–42 for both peptides.

Structure Calculation. NMR derived constraints for peptides **1** and **2** were used as input for a structure calculation as implemented in the software DYANA.[12] The 10/50 best scored structures of both peptides were then refined through energy minimization calculations with the program Discover (Biosym, San Diego). As expected from the qualitative analysis of NOESY spectrum, both peptides exhibited a pattern of backbone torsion angles consistent with an α-helical secondary structure from Pro[43] to Val[48] (Figure 2). Residues 41–49 are well-defined with root-mean-square (RMS) deviation for the backbone atoms of 0.40 Å for **1** and 0.42 Å for **2**. Also the side chains of these residues are almost defined (RMS deviations less than 1.5 Å). In a surface analysis of the ensemble of the refined structures of each peptide (Figure 3), residues Arg[41] and Arg[46] point to the same side of the helix, determining a positively charged area; while residues Phe[44], Val[47], and Val[48] form a

hydrophobic surface extending at the opposite side of the helix, thus forming an amphipatic structure. The residues at the N-terminal side of Arg[41] resulted to be less structured with a large degree of flexibility (RMS deviations above 2.0 Å for the backbone atoms). In this region there are not consistent structural elements in the calculated geometries for both peptides.

Structures Comparison. Conformational studies revealed that peptide **1** and the glycosylated analogue **2** adopted virtually indistinguishable structures in the water/HFA solution with minimal chemical shifts (Supporting Information) and NOE differences (Figure 1). A careful analysis of the whole data showed a significant difference between the temperature coefficients of the amide proton of Thr[33] (-4.62 in **1** vs -6.14 in **2**, Table 1) accompanied by a stronger NOE interaction between the amide proton resonances of Ala[32] and Thr[33] of **1** compared to that of **2** (Figure 1). These results can be explained assuming that a population of conformers of **1** adopts the Asx-turn conformation characteristic of the Asn-Xaa-Thr glycosylation sites.[13] The Asx-turn conformation is characterized by hydrogen bonding between the threonine amide proton and the carboxamide carbonyl of the asparagine side chain.[14] On the contrary, peptide **2** does not show any ordered conformation at the glycosylation site. Since the NMR analysis of glycopeptide **2** reveals no significant evidence of specific interactions between the peptide and the carbohydrate, it is likely that the lack of conformational tendency to the formation of an Asx-turn observed for the glycopeptide **2** results either from a steric effect in which the carbohydrate alters the conformational space available to the peptide or from a modulation of the local solvent structure that influences the environment experienced by the peptide. It has been reported[13] that peptides containing the glycosylation site sequence Asn-Xaa-Thr are subjected to conformational switching from the Asx-turn to the (type I) β-turn conformation upon the asparagine N-glycosylation. Actually, we do not observe the reduction of the temperature coefficient for Ala[32] and the enhancement of the NOE d$_{NN}$(Asn[31],Ala[32]) upon glycosylation, consistent with the absence of this turn.[13] Since both the active glycopeptide **2** and its unglycosylated counterpart **1** miss the first residue of the putative β-turn (corresponding to an N-terminal extension through position 29), this structure cannot be observed in our studies, leaving the N-terminal side of **2** unstructured.

In conclusion, a subtle conformational difference between the two peptides could explain their divergent immunological behavior. Nevertheless, the value of the
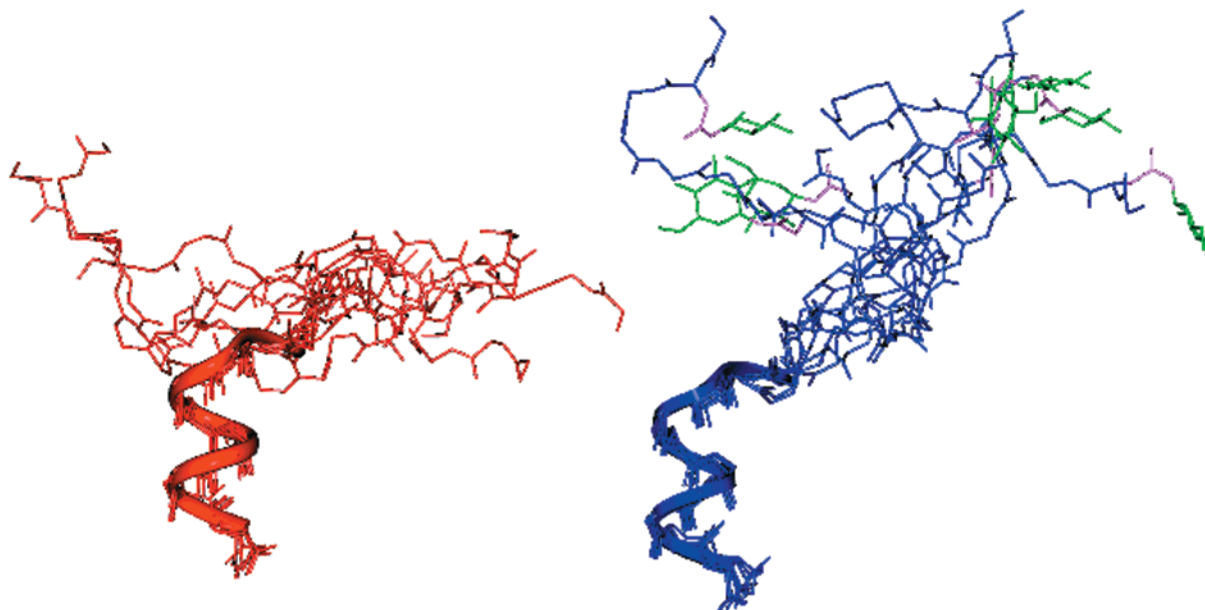
Figure 2. Superposition of the 10 optimized structures of peptides **1** (left) and **2** (right) with the C-terminal part at the bottom. Backbone heavy atoms (colored in red for peptide **1** and in blue for peptide **2**) are fitted from residue 41 to residue 49 (structured region), and this region is highlighted as an oval ribbon. For peptide **2**, the Asn[31] side chain is in pink and the β-glucose ring in green.
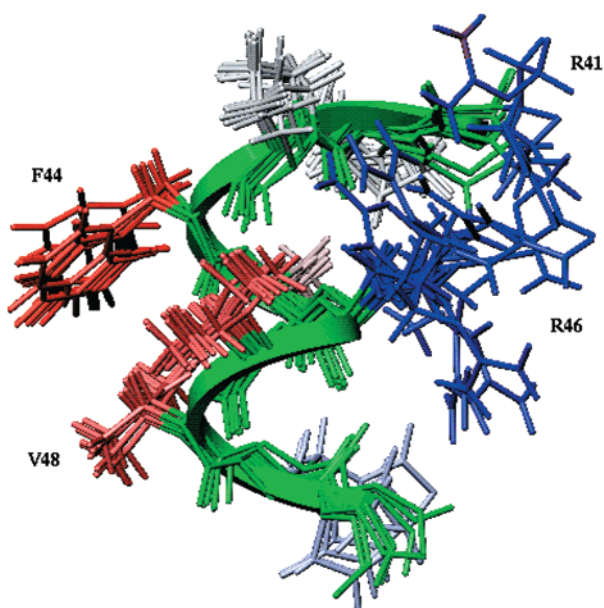

Figure 3. Superposition of the residues 41−49 (backbone heavy atoms) of the 10 optimized structures of peptide **2**. Amino acid side chains are colored according to their hydrophobic character. High, medium, and low polar residues are colored in pink, gray, and dark gray, respectively.

temperature coefficient, observed for the amide proton of Thr[33] in **1** (−4.62), indicates that the relevant hydrogen bond is quite weak and this region of the peptide **1** is prevalently in a random conformation, as indicated also by the molecular dynamic calculations. Thus, the specific antibody recognition of peptide **2** is most likely driven by direct interactions of the antibody binding site with the Asn-linked sugar moiety. We hypothesize that the peptide first binds the antibody through the interaction with the glycosylated asparagine and then the binding is stabilized by interactions with the well-defined amphipatic region of the peptide.

These results are relevant in view of the rational design of a peptidomimetic or non-peptide antibody ligand, to be used as a synthetic marker in the development of a diagnostic kit able to detect pathogenic autoantibodies in MS patient sera or as a new generation drug capable of specifically block circulating autoantibodies in patients affected by MS. Since the N-glycosylated asparagine is specifically recognized by the antibody, a designed synthetic antigen should retain this moiety, followed by a flexible link which joins it to an amphipatic, positively charged, α-helical mimetic structure.

Experimental Section

Sample Preparation. The peptides **1** and **2** were synthesized and purified as previously reported.[7] HFA and 99.9% D_2O were obtained from Aldrich (Milwaukee, WI), and [(2,2,3,3-tetradeuterio-3-(trimethylsilanyl)]propionic acid (TSP) was from MSD Isotopes (Montreal, Canada). The samples for NMR spectroscopy were prepared by dissolving the appropriate amount of **1** and **2** in 0.20 mL of ^1H_2O (pH 3.0), 0.05 mL of ^2H_2O, and 0.25 mL of HFA, obtaining a 1.5 mM solution for peptide **1** and a 1.0 mM solution for peptide **2**. TSP was used as internal chemical shift standard.

NMR Experiments. NMR spectra were recorded on a Bruker DRX-600 spectrometer. One-dimensional NMR spectra were recorded in the Fourier mode with quadrature detection, and the water signal was suppressed by a low-power selective irradiation in the homogated mode. Two-dimensional double quantum filtered correlated spectroscopy (DQF-COSY),[15] total correlated spectroscopy (TOCSY),[16] and nuclear Overhauser enhancement spectroscopy (NOESY)[17] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase increase of the initial pulse.[18] Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrixes were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was used for the TOCSY experiments. NOESY experiments were run at 300 K with mixing times in the range of 200−300 ms. The qualitative and quantitative analyses of DQF-COSY, TOCSY, and NOESY spectra were obtained using the interactive program package XEASY.[19] The temperature coefficients of the amide proton chemical shifts were calculated from TOCSY experiments

performed at different temperatures in the range 290−310 K by means of linear regression.

Structural Determinations and Computational Modeling. The nuclear Overhauser effect (NOE)-based distance restraints were obtained from NOESY spectra collected with a mixing time of 200 ms. The NOE cross-peaks were integrated with the XEASY program, and they were converted into upper distance bounds using the CALIBA program incorporated into the program package DYANA.[12] Cross-peaks which were overlapped more than 50% were treated as weak restraints in the DYANA calculation. An ensemble of 50 structures was generated with the program DYANA using 196 (intraresidue and sequential) and 62 (medium range) NOE-based distance constraints for peptide **1**, and 172 (intraresidue and sequential) and 44 (medium range) for peptide **2**. Two hydrogen bond restraints, suggested by the temperature coefficient values, were used for both peptides: CO Pro^{42} to NH Arg^{46} and CO Pro^{43} to NH Val^{47}. The 10 structures with the lowest value of the target function from the DYANA-generated sets for both peptides were subjected to extensive minimization. Steepest descents minimizations with 10 (kcal/mol)/Å^2 flatwell distance restraints were performed on all structures with the Discover 2.9 algorithm (Biosym, San Diego, CA) utilizing the consistent valence force field (CVFF).[20] Minimization proceeded until the change in energy was less than 0.05 kcal/mol. This was followed by unrestrained energy minimization until the change in energy was less than 0.01 kcal/mol. Since it is difficult to accurately simulate the physical−chemical properties of an HFA/water environment, we decided to minimize the impact of electrostatic interaction on the structures. The dielectric constant was set to 1, and formal charges were switched off. In this way, the efficacy of NOE distance restraints was maximized. The minimization effected an improved helical geometry and a lower total energy of the structures. The final structures were analyzed using the Insight 95.0 program (Biosym, San Diego, CA).

Supporting Information Available: ^{1}H NMR assignments of peptides **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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