Conformation−Activity Relationship of Designed Glycopeptides as Synthetic Probes for the Detection of Autoantibodies, Biomarkers of Multiple Sclerosis

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Sera from patients suffering from autoimmune disorders often contain multiple types of autoantibodies, some of which can be exclusive of a disease and thus used as biomarkers for diagnosis. Identification of these autoantibodies, as disease biomarkers, should be achieved using native antigens in simple biological assays. However, posttranslational modifications, such as glycosylation, may play a fundamental role for specific autoantibody recognition. In line with these observations, we described synthetic glycopeptides able to detect high autoantibody titers in sera of patients affected by multiple sclerosis, an inflammatory, demyelinating disease of the central nervous system. We describe here the conformation−activity relationship of a focused library of glycopeptides based on structural diversity, with the aim of defining the structural requirements for the interaction of these glycopeptide antigens with specific autoantibodies. The final goal is the optimization of an antigenic probe for multiple sclerosis, to be used in the development of a simple diagnostic test based on an immunoenzymatic assay. The reported results clearly indicate that glycopeptides able to reveal high antibody titers in multiple sclerosis sera are characterized by a type I′ β-turn around the minimal epitope Asn(Glc), which allows an efficient exposure of this moiety to antibodies interactions, in the context of a solid-phase immunoenzymatic assay.

Introduction

Among the many outputs of the 'omics' revolution indeed the characterization of a great number of new biomarkers has a relevant position. Biomarkers are 'decision-making tools' particularly important in the drug development process and, most importantly, in diagnosis. In fact, although the term biomarker originally refers to analytes in biological fluids, nowadays any measurement that predicts a person's disease state or response to therapy, and therefore has putative diagnostic and/or prognostic value, can be called a biomarker.[1] Consequently, the interest in any kind of biomarker is growing exponentially, boosted by hopes of immediate biomedical applications of the Human Genome Project, with pharmaceutical companies playing a central role, and several new 'biomarker companies' starting to offer their services. Moreover, focus in biomarker research is shifting from methods that can analyze one marker at a time to the so-called profiling methods, which allow the simultaneous measurement of a broad range of markers.[2]

Autoimmune disorders are very frequent and have a high social impact: thus, reliable diagnostic/prognostic tools are necessary for an early diagnosis and for monitoring disease activity. In some cases, there are therapies that can change the outcome and improve the quality of life, but an early diagnosis is absolutely necessary to halt disease progression. On the other hand, these therapies are expensive and not devoid of potentially serious side effects. The evaluation of their efficacy by means of specific prognostic assays has become a crucial issue. Sera from patients suffering from autoimmune disorders often contain multiple types of autoantibodies. Some autoantibodies can be exclusive of a disease and thus used as biomarkers for diagnosis; others fluctuate with disease exacerbations or remissions and are extremely valuable in the follow up of patients. Therefore, serial evaluations of autoantibodies from the quantitative and qualitative point of view are of utmost importance in the management of autoimmune disorders, in particular in evaluation of personalized therapeutic treatments.[3]

In this scenario, identification of autoantibodies, as disease biomarkers, should be achieved using native antigens in simple biological assays. However, growing evidence indicates that posttranslational modifications (i.e., acetylation, lipidation, citrullination, glycosylation, etc.), either native or aberrant, may play a fundamental role for specific autoantibody recognition in autoimmune diseases.[4] In line with these observations, we have recently described[5,6] synthetic glycopeptides able to detect high autoantibody titers in sera of patient affected by Multiple Sclerosis (MS), an inflammatory, demyelinating disease of the central nervous system (CNS).[7] The pathogenesis of MS has not been yet elucidated, but an autoimmune mechanism against myelin antigens is thought to contribute to the immunopathological mechanisms of the disease, even if the target antigens responsible for this inflammatory and demyelinating disease remain elusive. Different self-proteins have been investigated as potential targets for T or B cells in MS. The most extensively

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Table 1. Glycosylated and Unglycosylated Peptide Sequences

name (no.)	peptide sequence
[Asn^{31}(Glc)]hMOG(30−50) (**1**)	KN(Glc)ATGMEVGWYRPPFSRVVHL
hMOG(30−50) (**1′**)	KNATGMEVGWYRPPFSRVVHL
CSF114(Glc) (**2**)	TPRVERN(Glc)GHSVFLAPYGWMVK
CSF114 (**2′**)	TPRVERNGHSVFLAPYGWMVK
MBH36(Glc) (**3**)	RGKYTYN(Glc)GITYEGR
MBH36 (**3′**)	RGKYTYNGITYEGR
[Thr^{9}]CSF114(Glc) (**4**)	TPRVERN(Glc)GTSVFLAPYGWMVK
[Thr^{9}]CSF114 (**4′**)	TPRVERNGTSVFLAPYGWMVK
Ac-[*c*(Dap^{5},Asp^{10})]CSF114(Glc)[a] (**5**)	Ac-TPRV-*c*[Dap-RN(Glc)GHD]VFLAPYGWMVK
Ac-[*c*(Dap^{5},Asp^{10})]CSF114 (**5′**)	Ac-TPRV-*c*(Dap-RNGHD)VFLAPYGWMVK
Scramble CSF114(Glc) (**6**)	LAKVSYN(Glc)FRMETRVGWHPVGP
Scramble CSF114 (**6′**)	LAKVSYNFRMETRVGWHPVGP

[a] Dap: 2,3-diaminopropionic acid.

studied putative self-antigens are components of normal CNS myelin (myelin basic protein (MBP); proteolipid lipoprotein (PLP); myelin oligodendrocyte glycoprotein (MOG); etc.) or of posttranslationally modified forms of these myelin proteins.[8,9] It is now clear that some posttranslational modifications can create new self-antigens or even mask antigens normally recognized by the immune system. In particular aberrant glycosylations affect various parts of the immune response and have profound effects on immune tolerance.[4]

In light of the importance of specific sugars and conformation of epitopes involved in autoautoantibody recognition, in this work we analyze the conformation-activity relationship of a focused library of glycopeptides based on structural diversity. The aim of this study is to define the structural requirements for the autoantibodies/antigens interaction to be used as a model for further optimization of antigenic probes in MS. The optimized probe will be exploited in the development of a simple diagnostic test based on an immunoenzymatic assay (ELISA).[10]

Results

CSF114(Glc) as a New Antigenic Lead Compound. In a previous work,[11] we established the ability of the glucosylated analogue of the immunodominant epitope of MOG, [Asn^{31}(Glc)]-hMOG(30−50) (Entry **1** in Table 1), to detect autoantibodies in MS patients, but not in healthy controls, by solid-phase ELISA, while the unglucosylated analogue hMOG(30−50) (peptide **1′**) was inactive. We subsequently showed that the active glycosylated peptide **1** and the inactive unglycosylated peptide **1′** adopted similar solution conformations.[12] We concluded, therefore, that the ability of the former molecule to detect autoantibodies in MS was linked to characteristics other than conformation and that the specific autoantibody binding site on MOG glycopeptide **1** was related to the N-linked glucose moiety. This result, together with the observation that the N-glucosylated asparagine alone was able to bind MS autoantibodies in a solution-phase competitive ELISA experiment,[5] allowed us to determine the minimal epitope as the Asn(Glc) moiety. The previously reported extensive immunological analysis of the MOG glycopeptide and of several analogues[5] enabled us to optimize the exposure of the Asn(Glc) minimal epitope to the solvent and, consequently, to the antibodies, designing new antigenic probes to be used in the solid-phase conditions of an immuno-enzymatic assay, in which the antigen is adsorbed onto the plastic surface of the assay plate. As a proof of concept, to confirm that the amino acid sequence of hMOG(30−50) is not important per se, but provides an optimal presentation to the minimal epitope Asn(Glc), we designed a new antigenic peptide having the same amino acid composition as hMOG(30−50), but characterized by a β-hairpin structure (i.e. a β-turn flanked by two antiparallel β-sheet), in which the Asn(Glc) epitope was placed at the tip of the β-turn in an almost central position in the peptide sequence (position 7 over 21). The Asn-Gly sequence was chosen for the central residues of the turn since it is well-known that this sequence efficiently stabilizes type I′ β-turn.[13] Actually, Asn (position i+1, (L1)) and Gly (position i+2 (L2)) residues are found in the majority of the type I′ β-turn.[14] Residues flanking the putative β-turn were chosen to obtain good side chain interactions across strands. A Glu residue was placed in position −B2 faced to a Ser residue (position +B2) to allow H-bond formation and two Val residues were faced (positions −B3, and +B3) to optimize hydrophobic contacts. In this notation, B means that the residue is part of a β-strand (−B, first β-strand; +B, second β-strand), L is part of the β-turn.[14] A short stretch of hydrophobic residues (Trp^{18}, Met^{19}, Val^{20}) were placed at the C-terminal part of the sequence to anchor the peptide to the ELISA plate. The remaining residues were randomly selected among those of hMOG(30−50); the resulting sequence is shown in Table 1 (peptide **2**, termed CSF114(Glc), and the corresponding unglycosylated analogue **2′**).

The conformational behavior in solution of CSF114(Glc) (**2**) and its unglucosylated counterpart (**2′**) was investigated by means of circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy. CD and NMR spectra were recorded in water and in hexafluoroacetone (HFA)/water 50/50 v:v. This solvent mixture is a stabilizing agent which increases the intrinsic tendency of the amino acid sequence to fold in defined secondary structures.[15] It was also shown, in the same paper, that HFA molecules effectively provide a "teflon face" which secludes the peptide in a hydrophobic environment. Hence, this "Teflon face" could mimic the ELISA solid surface where a structural preorganization may occur. The secondary structure analysis of the CD data of **2** in water suggests the presence of disordered conformers with a relative minimum ellipticity at 195 nm. A shoulder at lower frequencies (around 215−220 nm) indicates the presence of low population of more ordered conformers. In HFA/water solution the shape of the CD spectrum suggests the presence of β-turns and/or β-strands folding with two minima at 215 and 205 nm (Supporting Information). Only minimal differences were observed in the spectroscopic data of **2′** compared to **2**; this issue will be discussed at the end of this section. The CD investigation described above was followed by the collection of a whole set of one- (1D) and two-dimensional (2D) NMR spectra. To check for the absence of an aggregation state of the peptides, spectra were acquired in the concentration range of 0.2−2 mM. No significant changes were observed in the distribution and in the shape of the ^{1}H NMR resonances, indicating that no aggregation phenomena occurred in this concentration range. Complete ^{1}H NMR chemical shift assign-

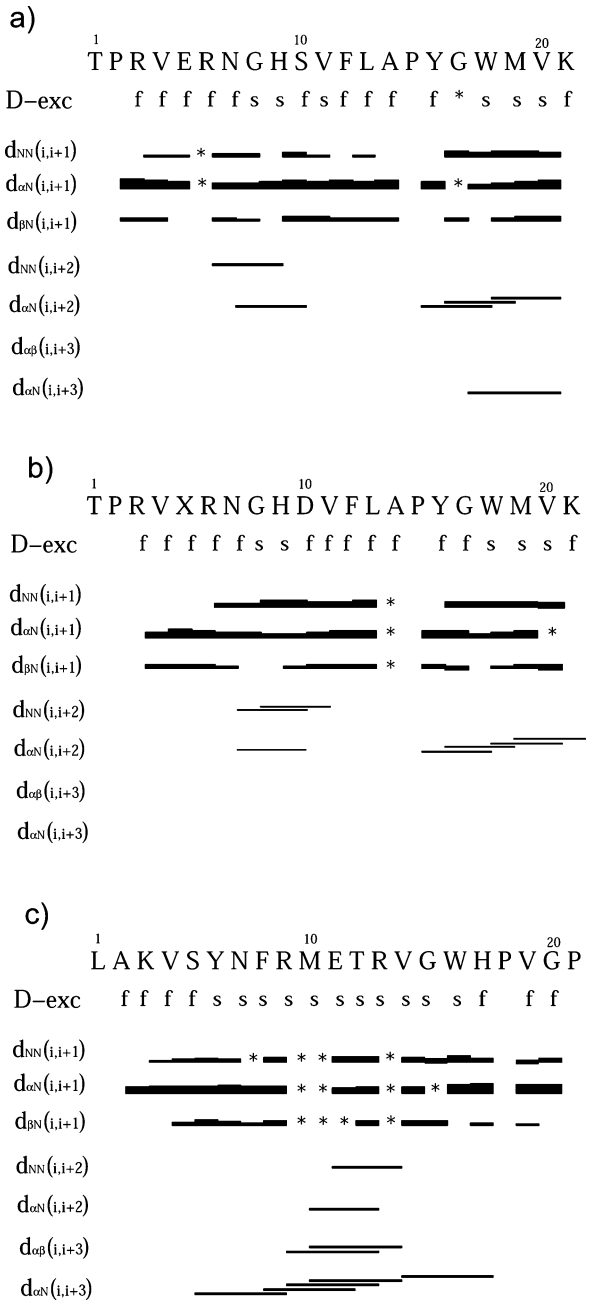

Figure 1. Summary of NMR derived experimental data of glycopeptides **2** (a), **5** (b), and **6** (c) in water/HFA 1:1 v:v solution. Dap[5] residue of **5** was denoted with X. D-exc: deuterium exchange rates (s, slow or moderately slow; f, fast). Schematic bar diagrams show the sequential and *medium* range connectivities observed in the NOESY spectra. Thickness of the bars is related to the NOE intensities. A star indicates signal overlapping.

ments were effectively achieved (Supporting Information) according to the Wüthrich procedure[16] via the usual systematic application of double quantum filtered correlated spectroscopy (DQF−COSY),[17] total correlated spectroscopy (TOCSY),[18] and nuclear Overhauser enhancement spectroscopy (NOESY)[19] experiments with the support of the XEASY software package.[20]

The whole NMR data confirmed the conformational behavior of **2** obtained by CD analysis in water and HFA/water solutions, showing a disordered state in the first environment, and a defined β-hairpin conformation in the latter one. In particular, NMR analysis of **2** was performed in water at pH 5 and at 300 K. Almost all NMR parameters indicate structural flexibility: $^3J_{HN-H\alpha}$ coupling constants are all within the range 6−8 Hz;

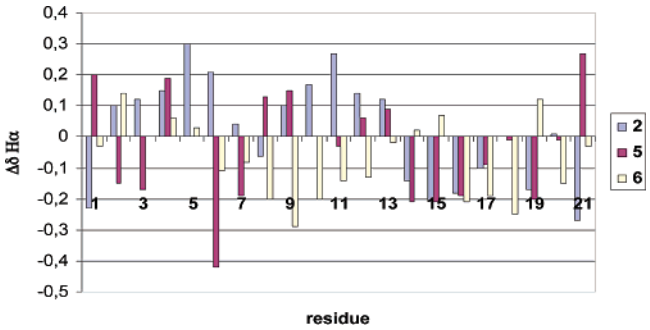

Figure 2. Plots of chemical shift deviations of H_α protons from random coil values in water/HFA 1:1 v:v solution. Random coil values were taken from ref 21b.

strong $d_{\alpha N}(i, i+1)$ nuclear Overhauser effects (NOE), which are generally observed in extended structures, appeared along the entire length of the peptide. A very weak $d_\alpha N(i, i+2)$ NOE observed between Asn[7] and His[9], together with the relatively low-temperature coefficient of His[9] NH ($|\Delta\delta/\Delta T| = 2.4$ ppb/K), and the downfield shift of the H_α resonances of the Gly[8] when compared to random coil reference values[21] (Supporting Information) indicate the presence of a structure population which possesses a β-turn moiety encompassing residues 6−9. H_α resonances of residues 14−17 also show downfield shifts indicating a propensity of this segment to the helical structures.

Regarding the HFA/water sample solution, the better dispersion of the proton resonances indicates a higher structural definition in this environment. The presence of a β-turn encompassing residues 6−9 is suggested by a weak H_α-NH_{i+2} connectivity between Asn[7] and His[9] and a strong NH−NH_{i+1} connectivity between Asn[7] and Gly[8] (Figure 1). A turn structure is supported by the observation of slowly exchanging NH at positions 8 and 9. A short stretch of antiparallel β-sheet involving residues 2−5 and 10−13 is inferred from some long-range NOE's including diagnostic NH−NH connectivity between residues 6 and 9. Further interstrand NOE contacts are observed between H_α of Val[4] and methyl protons of Val[11], H_α of Arg[3], and H_δ's of Phe[12], and methyl protons of Val[4] and H_α of Ser[10]. Other diagnostic interstrand NOE contacts could not be observed due to overlapping signals. The presence of a β-hairpin is supported by the observation of slowly exchanging NH at position 11.

Additional support for the β-hairpin is provided by the analysis of the H_α resonances which are strongly dependent on local secondary structure.[21] In fact, H_α's of Gly[8] exhibit an upfield shift of the NMR resonance, while Pro[2], Arg[3], Val[4], Glu[5], Arg[6], His[9], Ser[10], Val[11], Phe[12], and Leu[13] H_α signals exhibit significant downfield shifts (Figure 2). H_α resonances of residues from Ala[14] to Met[19] exhibit upfield shifts consistent with a helical structure along this peptide segment.

Hence, consistently with the CD results, NMR data indicate the presence of a β-hairpin in the region encompassing residues 2−13 with a turn at positions Asn[7]-Gly[8], while a helical structure is found at the C-terminal residues 14−19.

NMR data obtained for **2** in HFA/water solution were used as input for a restrained structure calculation using a simulating annealing protocol.[22] Figure 3a shows the 10 lowest energy conformers of the 200 calculated structures. Structures are well-defined both in regions 2−13 and in 15−19 with RMSD's of 0.4 and 0.3, respectively, calculated over the backbone atoms. A short stretch of antiparallel β-sheet involving residues 2−5 and 10−13 is observed together with a type I′ β-turn encompassing residues 6−9. Hence, the peptide shows a β-hairpin

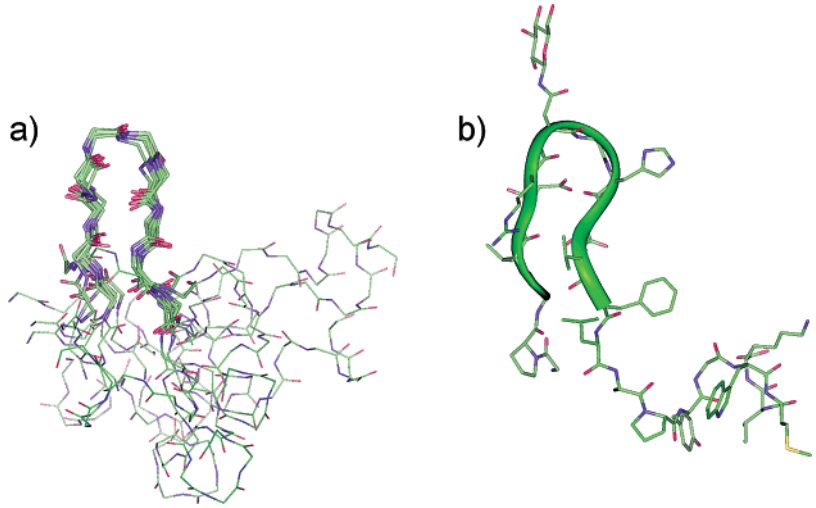

Figure 3. (a) Superposition of the 10 lowest energy conformers of **2**. Structures were superimposed using the backbone heavy atoms of residues 3−12 (thicker lines). Heavy atoms are shown with different colors (carbon, green; nitrogen, blue; oxygen, red; sulfur, gold). Side chains and hydrogen atoms are not shown for clarity. (b) Lowest energy conformer of **2**. β-hairpin structure is evidenced as a ribbon.

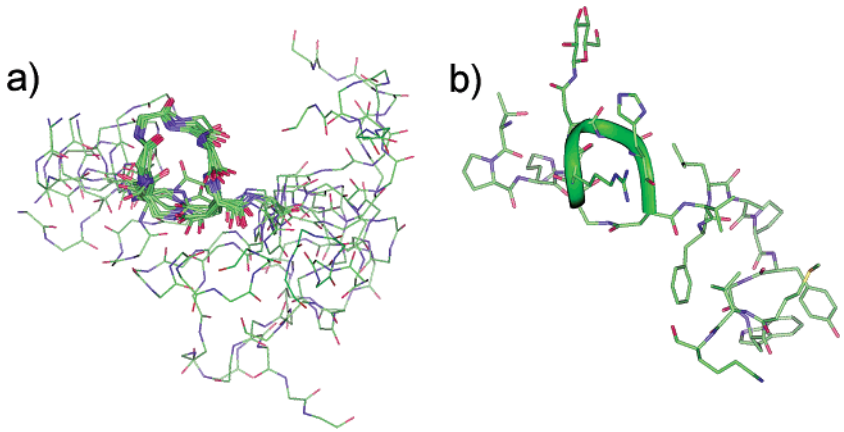

Figure 4. Superposition of the 10 lowest energy conformers of **5**. Structures were superimposed using the backbone heavy atoms of residues 5−10 (thicker lines). Heavy atoms are shown with different colors (carbon, green; nitrogen, blue; oxygen, red; sulfur, gold). Side chains and hydrogen atoms are not shown for clarity. (b) Lowest energy conformer of **5**. β-turn structure is evidenced as a ribbon.

structure with the Asn(Glc) moiety well exposed to the solvent and to possible intermolecular contacts (Figure 3b).

Unglycosilated peptide **2′** shows CD and NMR features very similar to its glycosylated analogue (Supporting Information). Detailed investigation on chemical shift difference of H_α's of the hairpin region (Supporting Information) indicates that this structure is slightly strengthened in **2** compared to **2′**. Consistently, the expected downfield shift of the Asn^7 H_α resonance upon glycosylation[23] is not observed moving from **2′** to **2** since it is compensated by the simultaneous stabilization of the turn structure. Cursory description of the design, along with very preliminary conformational data of **2**, was previously reported in a paper[5] describing in detail its immunological properties.

Glycopeptide Library Development. To verify that the β-hairpin structure is important for the glycopeptide activity, we synthesized and tested a small glycopeptide library (compounds **3**−**6**, Table 1) designed on the basis of structural diversity.[5]

Glycosylated MBH36 (3). The glycopeptide **3** (Table 1) was selected starting from the observation that its unglycosylated analogue (MBH36, **3′**) was shown by Perez-Paya et al.[14] to possess high population of type I′ β-hairpin structure in solution, through detailed CD and NMR analysis. The CD spectra of glycopeptide **3** performed both in water and HFA/water solutions were almost perfectly superposable with the corresponding ones of **3′** (data not shown). Therefore, glycopeptide **3** also shows a type I′ β-hairpin structure as its parent unglycosylated analogue **3′** and as the highly antigenic peptide **2**.

[Thr^9]CSF114(Glc) Derivative (4). The glucosylated Asn residue in the CSF114(Glc) sequence could mimic a glycosylation site of an endogenous MS myelin antigen(s). Therefore, we also tested a CSF114(Glc) analogue in which a Thr residue was placed in position 9 thus obtaining the Asn-Xxx-Thr consensus sequence of the protein glycosylation sites (peptides **4**, and **4′**, Table 1). CD analysis of the designed peptides **4** and **4′** also indicated the presence of a β-hairpin structure in HFA/water solution with spectra almost identical to those obtained for the parent peptide **2** (Supporting Information).

A Structurally Constrained Analogue: Ac-[c(Dap,^{5}Asp^{10})]-CSF114(Glc) (5). The design of cyclic structures to potentially enhance turn motifs is a well-established method.[24] Starting from the 3D structure of **2** (Figure 3), we replaced Glu^5 and Ser^{10}, whose side chains are spatially close in **2**, with 2,3-diaminopropionic acid (Dap) and Asp residues, respectively. Then, the side chains of the replacing residues were linked through an amide bond to obtain the cyclic analogues **5** and **5′** (Table 1). Glycopeptide **5** shows a different CD spectrum in HFA/water solution compared to that of **1** (Supporting Information). As in **2**, two minima can be observed in this spectrum, but at different wavelengths (202 and 218 nm). Therefore, we carried out a

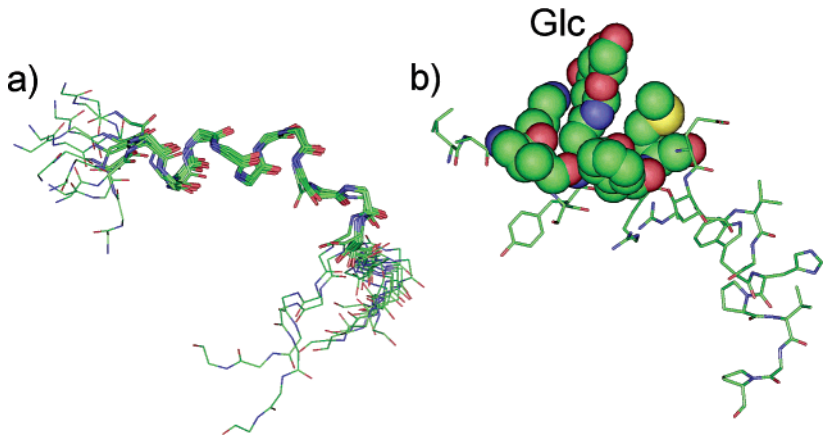

Figure 5. (a) Superposition of the 10 lowest energy conformers of **6**. Structures were superimposed using the backbone heavy atoms of residues 3−15 (thicker lines). Heavy atoms are shown with different colors (carbon, green; nitrogen, blue; oxygen, red; sulfur, gold). Side chains and hydrogen atoms are not shown for clarity. (b) Lowest energy conformer of **6**. Asn^{7}(Glc) and residues Lys^{3}, Val^{4}, Phe^{8}, and Met^{10} are shown as van der Waals surfaces.

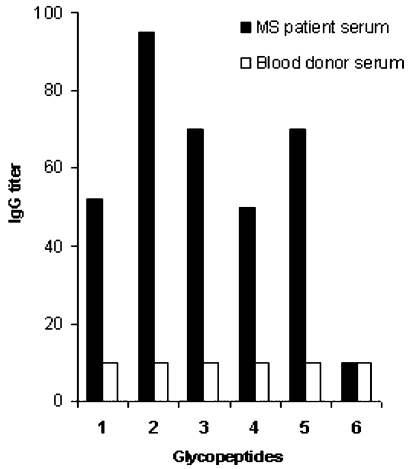

Figure 6. Autoantibody recognition in multiple sclerosis patients' sera. IgG titers to glycopeptides **1**, **2**, **3**, **4**, **5** and **6** in a representative serum, in comparison with blood donor serum. All the unglucosylated analogues **1′**−**6′** were inactive (data not shown).

detailed NMR and computational analysis of compound **5**. NMR parameters (Figures 1 and 2, and Supporting Information) indicate that the cyclic peptide **5** loses the hairpin structure.

In particular, NMR diagnostics for the β-strand regions are not observable. However, the d_{α}N(i, i+2) NOE between Asn^{7} and His^{9} indicates that a turn structure about residues 6−9 is still present, as in **2**. Structure calculation gave 52/100 conformations of **5** presenting a type I′ β-turn encompassing residues 6−9 (Figure 4a). As shown in Figure 4b, the Asn(Glc) moiety, which is included in the β-turn, is well exposed to the solvent. Virtually identical NMR parameters were found for unglycosylated analogue **5′** (Supporting Information).

A Scrambled Glycopeptide (6). To further confirm the importance of the secondary structure of the peptide antigens for a correct presentation of the minimal epitope in the autoantibodies recognition process in the context of the solid-phase ELISA, we also synthesized peptides with a scrambled sequence (**6** and **6′**, Table 1).

In this random sequence only the glucosylated Asn^{7} residue was left at its initial position in the sequence. A preliminary CD analysis (Supporting Information) indicates different conformational preferences of **6** compared to **2**. In particular, the two minima at 220 and 210 nm indicate a high tendency to the helix formation in **6**. NMR analysis confirmed this indication. Several NMR parameters (Figures 1 and 2, and Supporting Information) indicate the presence of an α-helix along residues 4−15 of **6**. Calculated structures (Figure 5a) showed the expected helical segment. Interestingly, in these structures part of the Asn^{7}(Glc) epitope is buried by Lys^{3}, Val^{4}, Phe^{8}, and Met^{10} side chains (Figure 5b). Again, glycosylation has only negligible effects on the conformational preferences of the peptides (Supporting Information).

Immunological Activity of Glycopeptides. The immunological activity of the designed glycopeptides **3**−**6** has been compared with that of the reference compounds **1** and **2** by solid-phase ELISA, using a new set of MS patients' sera (Figure 6). These data indicate that peptides **1**−**5** recognize high titers of specific autoantibodies in MS patients' sera. In contrast, lack of antigenic activity of peptide **6** can be observed.

Discussion

In light of the importance of the conformation and of the correct exposure of epitopes involved in autoantibody recognition, we have recently studied synthetic glycopeptides able to recognize autoantibodies in MS patients' sera in an optimized solid-phase immunoenzymatic assay. In particular, we described the glycopeptide CSF114(Glc), the first antigenic probe accurately measuring specific autoantibody titers in the sera of a patient population, as a disease biomarker.[5] The potential of CSF114(Glc) as a probe for MS-biomarkers in the clinic is based on the good correlation between the detected levels of MS autoantibodies and the severity of the disease or the prediction of reoccurrence.[6] In fact, CSF114(Glc) is able to recognize, by ELISA, the presence of specific autoantibodies in the sera of a significant proportion (30%) of MS patients, and with high specificity for this disease (94%), i.e., no false positive results were obtained with sera from patients with other autoimmune diseases or with other infective neurological diseases. Autoantibodies specific for CSF114(Glc), isolated from MS patients' sera, recognized myelin and oligodendrocyte antigens by immunohistochemistry, but did not stain other nonrelevant tissues.[5] We demonstrated that CSF114(Glc) is a reliable, specific probe in a longitudinal study of untreated MS patients.[5] Development of anti-CSF114(Glc) antibodies paralleled clinical activity and brain lesions positive to MRI. Taken together, these data confirmed the significance of synthetic modified peptides as probes to detect specific autoantibodies, biomarkers of MS.

This study describes the conformation−activity relationship of CSF114(Glc) (**2**) and of a focused library of glycopeptides (**3**−**6**) as potential antigenic probes for MS. The design of these

peptides started from the observation that the minimal epitope of the originally described antigenic glycopeptide [Asn^{31}(Glc)]-hMOG(30−50) (**1**) is the Asn(Glc) moiety.[5,12] However, we observed that a good antigenic activity of synthetic glycopeptides containing such minimal epitope depends on an optimal exposure on the plastic surface of the assay plate. To optimize this fundamental aspect, we developed the conformation−activity relationship study reported in the present paper. We found that the new glycopeptides **2**-**4** possess a β-hairpin motif encompassing residues 2−13. The β-hairpin is composed by a type I′ β-turn about residues 6−9 and two extended flanking regions (2−5 and 10−13) which form a short antiparallel β-sheet (Figure 3). The minimal epitope Asn(Glc) is placed at position i+1 of the β-turn, nicely exposed to antibody recognition. The cyclic analogue **5** also shows the type I′ β-turn motif (Figure 4). Even if the extended flanking region of the β-hairpin are lost in this structure, Asn(Glc) moiety is still well exposed for intermolecular interactions. The optimal conformational propensity of these peptides correlates well with their good antigenic activity (Figure 6). Unglycosylated analogues **2′**−**5′**, though showing very similar structural preferences compared to the glycosylated counterparts, are not antigenic, confirming that the autoantibodies specifically recognize the N-linked glucose moiety. Finally, the inactive scrambled glycopeptide **6** does not show any propensity to the β-turn/ β-hairpin formation in solution. An α-helix encompassing residues 4−15 is observed in the calculated structures of **6** (Figure 5a). In these structures the epitopic Asn^7 side chain, including the amide NH group, is deeply buried by Lys^3, Val^4, Phe^8, and Met^{10} side chains. In contrast, glucose moiety is exposed to the solvent (Figure 5b). This is in line with the finding that an Asn N-linked glucoside, and not the sugar per se, is fundamental for the antibody interaction.[5] Of course, also the unglycosylated analogue **6′**, similarly to the other unglycosylated peptides, does not bind MS autoantibodies.

The observed conformational preferences of the designed peptides **2**−**6** can explain their different antibodies affinities. Glycopeptides **2**−**5**, all possessing the type I′ β-turn motif around the minimal epitope Asn(Glc) and, therefore, enable an optimal exposure required for intermolecular interactions of this epitope with the autoantibodies and results in detection of increased titers in the solid-phase ELISA, when compared to the unstructured lead compound **1** and to the scrambled peptide **6**.[5] It appears, therefore, that the β-turn conformation of these glycopeptides is fundamental for a correct exposure of the epitope containing Asn(Glc). In fact, in the glycopeptide **6** the Asn(Glc) group is hidden to the solvent, and, hence, it is not available for interacting with MS autoantibodies. Since active glycopeptides **2**−**5** revealed similar antibody titers (Figure 6), the amino acid composition of the hairpin structure appears to play a minor role, if any, in the autoantibodies recognition.

Interestingly, the response to CSF114(Glc) was limited to the IgG2 subclass,[6] a subclass characteristic of a T-cell independent response to glycoepitopes and rarely found, until now, particularly in MS.[25−27] From this point of view, bacterial products, such as those containing polysaccharides, were detected in brain lesions and linked to B and T cell stimulation[28] and activation of inflammatory responses.[29] In addition, it has already been shown that B cell activation in MS may be dependent on bacterial as well as viral antigens.[30] An increasing number of mimicries between bacterial proteins and myelin antigens have been documented, suggesting a pathogenetic link between the antibody responses to bacterial carbohydrate products and those to myelin antigens.[31,32] In this context, synthetic modified peptides are indeed very powerful tools to investigate antigenic mimicry and, most importantly, to obtain specific probes that will enable the discovery of new biomarkers of autoimmune diseases, which are not detectable by the proteomic approach.[33]

Conclusions

In this work we describe the conformation−activity relationship study of a small focused library of glycopeptides based on structural diversity. Globally, our results confirm the role of glycosylation and, most importantly, of conformation in recognition and binding to MS autoantibodies in the solid-phase context of an immunoenzymatic assay (ELISA). Glycopeptides, which reveal high antibody titers in MS sera, all show a type I′ β-turn around the minimal epitope Asn(Glc), which allows an efficient exposure of this moiety to autoantibodies interactions. The results of this study will be used as a model for further optimization of antigenic probes in MS. The optimized probe will be then introduced in a simple diagnostic test in the ELISA or other biosensor-based formats. Patients with MS will benefit from early diagnosis, better prognosis, optimized therapy, and more effective monitoring.

Experimental Section

Sample Preparation. The peptides **1**−**6** and **1′**−**6′** were synthesized and purified as previously reported.[5,11] HFA and 99.9% D_2O were obtained from Aldrich (Milwaukee, WI), and [(2,2,3,3-tetradeuterio-3-(trimethylsilanyl)]propionic acid (TSP) from MSD Isotopes (Montreal, Canada).

Circular Dichroism. All CD spectra were recorded using a JASCO J810 spectropolarimeter, with a cell of 1 mm path length. The CD measurements were performed using a measurement range from 260 to 190 nm, 1 nm bandwidth, 8 accumulations, and 10 nm/min of scanning speed, at room temperature. We performed comparative CD spectra at pH = 5 and pH = 7 of all the analyzed peptides and these were virtually indistinguishable. The pH of the aqueous sample was adjusted by adding small amount of HCl to phosphate buffer solution. Peptides concentration was determined spectrophotometrically using tyrosine and tryptophan absorbance as described.[34] For all peptides, 300 μL of a 1.0 mM water stock solution was prepared and the final 0.1 mM solutions were obtained adding 30 μL of the stock solution to the appropriate amount of buffered water and HFA. Mean residue ellipticities were calculated for each sample by the usual method.[35]

NMR Spectroscopy. The samples for NMR spectroscopy were prepared by dissolving the appropriate amount of peptides in 0.20 mL of ^1H_2O (pH 5.0), 0.05 mL of ^2H_2O, and 0.25 mL of HFA, obtaining 1−2 mM solutions. TSP was used as internal chemical shift standard. NMR spectra were recorded on a Bruker DRX-600 spectrometer. All the spectra were recorded at a temperature of 300 K. The spectra were calibrated relative to TSP (0.00 ppm) as internal standard. 1D-NMR spectra were recorded in the Fourier mode with quadrature detection. The water signal was suppressed by the hard pulse WATERGATE scheme. 2D DQF−COSY,[17] TOCSY,[18] and NOESY[19] spectra were recorded in the phase-sensitive mode using the method from States.[36] Typical 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 with mixing times in the range of 150−300 ms. The qualitative and quantitative analyses of DQF−COSY, TOCSY, and NOESY spectra were obtained using the interactive program package XEASY.[20] $^3J_{HN-H}\alpha$ coupling constants were obtained from 1D ^{1}H NMR and 2D DQF−COSY spectra. The temperature coefficients of the amide proton chemical shifts were calculated from 1D ^{1}H NMR and 2D DQF−COSY experiments

performed at different temperatures in the range 300−320 K by means of linear regression.

Structural Determinations and Computational Modeling. The 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 were converted into upper distance bounds using the CALIBA program incorporated into the program package DYANA.[22] Cross-peaks which were overlapped more than 50% were treated as weak restraints in the DYANA calculation. In a first step only NOE derived constraints (Supporting Information) were considered in the annealing procedures. For each examined peptide, an ensemble of 200 structures was generated with the simulated annealing of the program DYANA. An error-tolerant target function (tf-type=3) was used to account for the peptide intrinsic flexibility. Nonstandard Asn(Glc) residue was added to DYANA residue library using MOLMOL.[37] 50/200 structures were chosen, whose interprotonic distances best fitted NOE derived distances, and then refined through successive steps of restrained and unrestrained energy minimization calculations using the Discover algorithm (Accelrys, San Diego, CA) and the consistent valence force field (CVFF).[38] The minimization lowered the total energy of the structures; no residue was found in the disallowed region of the Ramachandran plot. The final structures were analyzed using the InsightII program (Accelrys, San Diego, CA). Graphical representation were carried out with the InsightII program (Accelrys, San Diego, CA). RMS deviation analysis between energy minimized structures were carried out with the program MOLMOL.[37] The PROMOTIF program was used to extract details on the location and types of structural secondary motifs.[39]

ELISA. Antibody titers were determined by solid-phase ELISA as previously described.[5] Briefly, 96-well activated polystyrene ELISA plates were coated with 1 μg/100 μL/well of peptides in carbonate buffer 0.05 M (pH 9.6) and incubated at 4 °C overnight. After three washes with saline solution containing 0.05% Tween 20, nonspecific binding sites were blocked by 10% fetal calf serum (FCS) in saline Tween (100 μL/well) at room temperature for 60 min. Serum diluted from 1:100 to 1:100.000 were applied at 4 °C for 16 h in saline Tween 10% FCS. After five washes, we applied 100 μL/well of alkaline phosphatase-conjugated anti human IgG Fab2-specific affinity purified Ab, diluted 1:500 in saline Tween/FCS. After an overnight incubation and five washes, we applied 100 μL of substrate solution consisting of 2 mg/mL *p*-nitrophenyl phosphate in buffer 10% diethanolamine. The reaction was blocked after 30 min with 50 μL of 1 M NaOH and the absorbance read in a multichannel ELISA reader at 405 nm. The antibody levels are expressed as titer (sample dilution which reaches the average plus three standard deviations of blanks).

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Supporting Information Available: CD spectra and NMR data of the analyzed peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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