J|A|C|S COMMUNICATIONS

Published on Web 03/31/2010

Effect of Glycosylation on *Cis*/*Trans* Isomerization of Prolines in IgA1-Hinge Peptide

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Proline has a unique propensity for the *cis*/*trans* isomerization of the peptide bond linking to the previous amino acid residue, which plays a role as determinants of tertiary structure of proteins.[1] In short flexible peptides, the *cis* conformation accounts for 10−30% of the total prolines,[2] and this ratio significantly depends on the amino acid type, as well as modification, of the preceding residue.[3]

Mucin-type *O*-glycan, a post-translational modification found in many glycoproteins, plays an important role in many biological functions. The *O*-glycosylation of Ser or Thr significantly affects the peptide backbone conformation, as revealed by NMR-based analyses.[4]

Human serum immunoglobulin A1 (IgA1), different from other serum immunoglobulins, contains *O*-glycans in its hinge region. The amino acid sequence of the hinge peptide (HP) is mostly composed of Ser, Thr, and Pro (VPSTPPTPSPSTPPTPSPS) and naturally possesses five *O*-glycans.[5]

It was reported that the arrangement of the Fab and Fc fragments of IgA1 was quite different from that of IgA2, which have neither HP nor *O*-glycan.[6] This suggests that the difference of tertiary structures of the two isotypes may be attributed to the *O*-glycosylation in the hinge peptide. In this study, we analyzed the structure of HP of IgA1 by NMR, especially focusing on the effects of the glycosylation on the *cis*/*trans* isomerization of prolines.

HP labeled with ^{13}C and ^{15}N at the Pro5, Pro8, Pro10, Pro13, or Pro16 residue was synthesized by an Fmoc method (see Materials and Methods, Table S1 in Supporting Information). $^{1}H-^{13}C$ HSQC spectra of HP contain the major and minor sets of resonances (Figures 1, S1). Analysis of 2D NMR spectra revealed that major and minor sets were assigned to the *trans* and *cis* conformers, respectively (Figure S2). The *cis*/*trans* ratios in HP are ∼10% (Figure 1 and Table 1), which are comparable to earlier studies.[2]

We prepared the multiple *N*-acetylgalactosamine (GalNAc)-glycosylated-HP (glyco-HP) by polypeptide-*N*-acetyl-galactosyl-transferase-2 and -10. The glycosylation sites are shown in Figure 1 and Table S1. After the glycosylation, the *cis*/*trans* ratios decreased to 2−3% (Figure 1, Table 1). It is noticeable that the degree of decrease for Pro13 is relatively small and that the preceding residue, Thr12, is not glycosylated (Figure 1 and Table S1).[5b] This demonstrates that the *cis*/*trans* ratio of Pro is lowered by the glycosylation at the preceding Ser/Thr. On the other hand, the decrease in Pro13 may be induced by a relatively long-range effect, as described later.

The *cis*/*trans* ratio of HP and glyco-HP were analyzed at various temperatures, and relevant ΔG values were calculated (Figure S3).

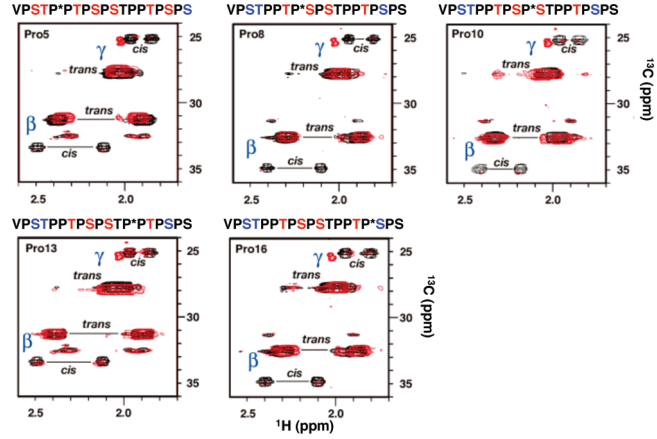

Figure 1. Overlay of $^{1}H-^{13}C$ HSQC spectra of HP with (red) or without (black) *O*-glycosylation. In the peptide sequences shown above the panels, the isotope-labeled Pro (asterisk) and fully (red) and partially/alternatively (blue) glycosylated Ser/Thr in glyco-HP are marked.

Table 1. Thermodynamic Parameters for Prolyl *Cis*/*Trans* Isomerization

		Pro5	Pro8	Pro10	Pro13	Pro16
ratio[a]	HP	0.126	0.092	0.088	0.115	0.095
	glyco-HP	0.022	0.027	0.015	0.051	0.033
	fold decrease	5.7	3.5	6.0	2.3	2.9
ΔG^a ($kJmol^{-1}$)	HP	4.9	5.9	5.9	5.3	5.8
	glyco-HP	9.7	9.8	10.4	7.7	9.1
	$\Delta\Delta G$	4.8	3.9	4.5	2.4	3.3
ΔH^b ($kJmol^{-1}$)	HP	2.3	7.3	4.5	3.1	6.3
	glyco-HP	16.7	22.2	18.8	10.8	18.9
	$\Delta\Delta H$	14.5	14.9	14.3	7.7	12.5
ΔS^b ($kJmol^{-1}$)	HP	−0.009	0.006	−0.004	−0.007	0.003
	glyco-HP	0.027	0.046	0.031	0.013	0.037
	$\Delta\Delta S$	0.036	0.041	0.036	0.02	0.034

[a] Values at 288 K. [b] Obtained by van't Hoff plot for data at 278−323 K.

van't Hoff analyses were applied to obtain ΔH and ΔS (Table 1). Since both the $\Delta\Delta H$ and $\Delta\Delta S$ values upon glycosylation are positive in all the cases, $\Delta\Delta H$ contributes to the positive $\Delta\Delta G$. Namely, the effect of glycosylation on reducing the *cis*/*trans* ratio is enthalpy-driven. It should be also noted that $\Delta\Delta H$ values for Pro5, Pro8, and Pro10 are very close to one another. All the above suggest a possible common pattern of an attractive interaction, such as a hydrogen bond, between the sugar and peptide moieties, occurring only in the *trans* conformation. We performed a hydrogen−deuterium exchange experiment and observed retardation in the exchange rate of the sugar amide proton of GalNAc-Thr in glyco-HP, compared with benzylGalNAc and GalNAc at ∼20-fold (Figure S4). This indicated the formation of a hydrogen bond

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10.1021/ja9106429

involving the sugar amide proton in the dominant *trans* conformation. Indeed, direct or water-mediated hydrogen bonds between the GalNAc amide and peptide backbone are observed for other glycopeptides.[7]

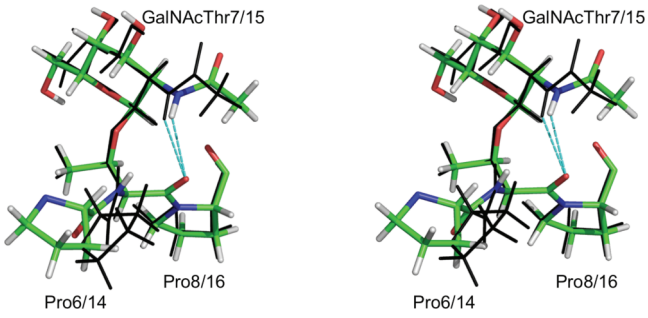

Figure 2. NMR structure of the major conformation of glyco-HP (stereo). Pro6-GalNAcThr7-Pro8 (stick) and Pro14-GalNAcThr15-Pro16 (wire) are shown, where hydrogen bonds between GalNAc and peptide backbone are shown by cyan lines.

We calculated the structure of the major conformer of glyco-HP using $^{1}H-^{1}H$ distances obtained by NMR (Figures 2, S5, S6). Although the initial stage of the structure calculation did not contain hydrogen bond constraints, probable hydrogen bonds between GalNAc amide NH and Thr backbone O are observed in most of the structures. Therefore, relevant hydrogen bond constraints are included in the final calculation. This hydrogen bond pattern is consistent with previous NMR observations for other glycopeptides.[7a,b] The calculated structures are well converged at least around the GalNAcThr residues (Figure S6), and the conformations of Pro6-GalNAcThr7-Pro8 and Pro14-GalNAcThr15-Pro16 are very similar to each other (Figure 2). It should be noted that the observed hydrogen bonds largely restrict the ψ angle of the peptide backbone to $\sim$155° in Figure 2. It is likely that this angle is not suitable for the *cis* conformation of the following Pro; i.e., the α-proton of Thr and carbonyl carbon of Pro will be too close, with a distance of $\sim$2.5 Å, if the *cis* conformation is imposed (Figure S7). We, therefore, propose the "short-range" effect of glycosylation on the *cis*/*trans* ratio of the following Pro, which is consistent with the thermodynamic parameters that predict the existence of hydrogen bond(s) only in the *trans* conformation.

It should be noted that the $\Delta\Delta G$ value for Pro13 is nearly half as large as that for Pro5 (with similar sequential context), although Thr12 is not glycosylated (Table 1).[5b] To explain this, we should consider relatively "long-range" sugar−peptide or sugar−sugar interactions. NMR analyses of highly glycosylated mucin-type glycopeptides revealed a propensity for extended conformations.[8] In the structural models, extensive sugar−sugar or sugar−peptide contacts are expected between residues that are not adjacent to each other, which may contribute to the propensity. Indeed, NOE contacts between separate residues were observed for other mucin-type glycopeptides,[9] as well as in this study (Table S2). Since such a conformational propensity is likely to shift the *cis*/*trans* equilibrium, a decrease in the ratio should be attributed to a combination of the "short-" and "long-range" effects, although the explicit structural feature for the latter is yet unknown.

The uniformity of the proryl peptide bond observed in the present study will lead to reducing the conformational variety and flexibility of the natural glycosylated HP of IgA1. Namely, considering the five proline residues analyzed in the present study, for example, the possibility that all of these exist in the *trans* conformation increases from 58% to 87% upon glycosylation. In addition, the structure calculation revealed that glycosylation significantly improves the structural convergence of the peptide backbone of the *trans* conformer (Figure S6), which is likely to be attributed to the hydrogen bonding and "long-range" interactions, as discussed above. The decrease in the conformational variety of IgA1-HP upon glycosylation probably leads to a decrease in the variety of relative orientations between Fab and Fc regions.

An abnormal *O*-glycosylation in the HP of IgA1 has been implicated in the pathology of IgA nephropathy.[10] The glycosylation defect of the HP possibly affects the ensemble in the relative orientations of Fab and Fc. This may, hence, affect the formation of the IgA aggregate related to the pathogenesis of IgA nephropathy.

Acknowledgment. This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO).

Supporting Information Available: Methods regarding sample preparation and NMR analyses, NMR spectra, van't Hoff plots, hydrogen−deuterium exchange experiments, and structure ensembles of glyco-HP and HP. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA9106429