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Conformational Aspects of Boc–Phe–Pro–Asn–Ala–Thr–NHMe; A Model of a *N*-Glycosylation Site in Ovomucoid

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ABSTRACT: A peptide sequence (Boc–Phe–Pro–Asn–Ala–Thr–NHMe) corresponding to one of the *N*-glycosylation sites in hen egg ovomucoid was studied with its related fragment analogues, Boc–Pro–Asn–Ala–Thr–NHMe, Boc–Asn–Ala–Thr–NHMe, and Boc–Ala–Thr–NHMe by NMR and CD to investigate their backbone conformations and possible intramolecular interactions. From the CD study, Boc–Phe–Pro–Asn–Ala–Thr–NHMe may adopt some ordered structures in aqueous solution. However, in this peptide series, there is no stable hydrogen bonds involving backbone amide protons nor is there any definitive evidence for intramolecular hydrogen bonds involving Asn and Thr side chains. But, it is evident from the temperature study of chemical shifts that the hydrogen bonding tendency of the Thr hydroxyl protons and the Asn carboxyamide protons is dependent on both the triplet sequence and chain length of the peptide analogues. The potential to form some kind of loop structure rather than the presence of some stable secondary structure is indicated.

KEY WORDS NMR / Circular Dichroism / Triplet Sequence / Ovomucoid / Intramolecular Interaction /

In Asn linked glycoproteins, carbohydrates are covalently bound to Asn residues located within the so-called triplet sequence, –Asn–X–Ser(or Thr)–.[1] This sequence with X standing for any arbitrary amino acid other than Pro, has been shown to be necessary for glycosylation.[1–3] There are two proposals for this enzymatic glycosyl transfer mechanism and both involve Ser(or Thr) side chains in intramolecular interactions with the Asn residue. The first, proposed by Marshall on the basis of a space filling molecular model, has an intramolecular hydrogen bond between the carbonyl oxygen of the Asn side chain and hydroxyl hydrogen of Ser(or Thr).[1] The other proposed by Bause and Legler, is a hydrogen bond between the carboxyamide hydrogen of Asn and hydroxyl oxygen of Ser(or Thr).[4] The latter was derived from enzymatic *N*-glycosylation of model peptides and recently supported by NMR studies of the tripeptide, Boc–Asn–Gly–Ser–OMe.[5] Besides such a side chain interaction, from the study of enzymatic *N*-glycosylation of peptides with restricted conformation due to disulfide bridges, it has been suggested that an appropriate backbone conformation is necessary for *in vitro N*-glycosylation.[6] Also, the fact that the glycosylation rate of –Asn–X–Ser(or Thr)–peptides modeling natural glycopeptide backbones increases with chain length suggests the existence of some preferential conformation.[7] Conformation sufficient for *N*-glycosylation is believed to be something akin to some kind of folded or loop structure. In fact, it has been proposed that, from statistical analysis of various amino acid sequences, the glycosylated Asn residues are frequently located within β-

turn regions.[8,9]

Several attempts have been made to elucidate the backbone conformation of peptides containing triplet sequences. From CD spectroscopy, it has been proposed that peptides which could be glycosylated adopt some spatially organized structure.[7,10] Conformational energy calculation of Ac–Asn–Ala–Thr–NH_2 has shown that these tripeptides have a strong tendency to adopt characteristic folded structures stabilized by hydrogen bonding between Asn and Thr side chains.[11] There is, however, no definite evidence to show that the triplet sequence adopts the β-turn or any other secondary structure.

In this article, because short-range interactions contribute to the stabilization of β-turn structures,[12,13] Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its related fragment analogues were synthesized to investigate the conformational aspects of peptides containing the triplet sequence. The sequence –Phe–Pro–Asn–Ala–Thr– is one of the four major sites of carbohydrate attachment (8—12 fragment) in hen egg ovomucoid.[14] Pro residues are favorable for the formation of β-turn structures due to their rigid backbone structures,[15] and the formation of β-turn structures with a –Pro–Asn– sequence as the corner region has been observed for both linear[16] and cyclic peptides.[17] Therefore, the peptide Boc–Phe–Pro–Asn–Ala–Thr–NHMe is expected to adopt a β-turn structure.

EXPERIMENTAL

Materials

All peptides were synthesized by the dicyclohexylcarbodiimide-*N*-hydroxysuccinimide method except in the case of coupling Asn to Ala where the *p*-nitrophenyl ester method was used. Conversion of Thr to its methylamide was done by *N*-methylamine in methanol as described.[18] The peptides were purified through repetitive recrystallizations and identified by ^{1}H NMR.

Method

^{1}H NMR spectra were recorded on JEOL FX-270 and GX-500 spectrometers operated at 270 and 500 MHz, respectively. Hexadeuterated dimethylsulphoxide (DMSO-d_6) and deuterium oxide (^2H_2O) were used as solvents. A $H_2O/^2H_2O$ 9/1 (v/v) mixture was also used to observe amide proton resonances in aqueous solution. The sample concentration was 0.03 M for all solvent systems and the sample temperature was approximately 26°C. Tetramethylsilane (TMS) and sodium 2,2,3,3-tetradeutero-3-trimethylsilyl propionate (TSP) were used as internal standards in DMSO-d_6 and in aqueous solutions, respectively. ^{1}H–^{1}H spin coupling constants were reported in Hz and the digital resolution was 0.31—0.33 Hz/point.

^{13}C NMR spectra were recorded on a JEOL PS-100 spectrometer operated at 25.15 MHz. The chemical shift was reported from external TMS without calibration. The sample concentration was 0.2 M and the sample temperature, 32°C.

CD spectra in aqueous solutions were recorded on a JASCO J-20A spectrometer. The path-length was 0.2 mm and the sample temperature 26—28°C. The sample concentration was in the range of 10^{-3}—10^{-4} M. The data were expressed in molar ellipticity, (θ), in units of deg m^2 mol^{-1}.

RESULTS AND DISCUSSION

Circular Dichroic Study

The CD spectra of the peptide series in aqueous solutions at neutral pH are shown in Figure 1. The spectrum of Boc–Phe–Pro–Asn–Ala–Thr–NHMe, characterized by a negative band at 237 nm, a positive band at 224 nm, and a relatively intense negative band at 210 nm is distinctly different from the spectra of Boc–Pro–Asn–Ala–Thr–NHMe and Boc–Asn–Ala–Thr–NHMe. The latter peptides are considered to be in a disordered form due to a characteristic negative band below 210 nm.[19] How-

ever, the spectrum of Boc–Phe–Pro–Asn–Ala–Thr–NHMe may support the presence of some ordered structures in this peptide in aqueous solution. The positive band at 224 nm may be due to the Phe chromophore as Ac–Phe–NHMe gives a CD band at 215—225 nm in aqueous and dioxane solutions.[20,21] Although the contribution of the Phe chromophore prevents a more definitive conclusion, the negative band at 237 nm suggests a γ-turn structure. The intramolecular 3-1 hydrogen bonded Pro shows a negative band at 220—230 nm as found in the γ-turn structure of thyrotropin releasing factor.[22] Also the spectrum of Boc–Phe–Pro–Asn–Ala–Thr–NHMe is similar to that of Phe–Ser–Pro–Phe–Arg which is believed to contain a γ-turn structure.[23] The spectrum is also similar to that of Piv–Pro–Aib–NHMe in trifluoroethanol which is considered to be a mixture of type I (III) and II β-turn structure from NMR data.[24]

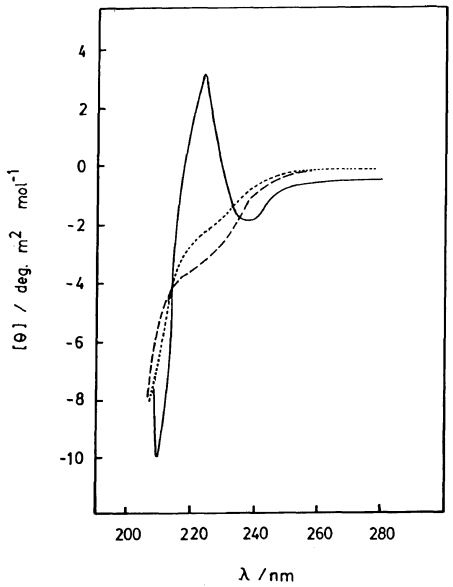

Figure 1. CD spectra in aqueous solution at pH 7. (——) Boc–Phe–Pro–Asn–Ala–Thr–NHMe; (– – – –) Boc–Pro–Asn–Ala–Thr–NHMe; (------) Boc–Asn–Ala–Thr–NHMe.

As CD spectra corresponding to β- and γ-turn structures are somewhat similar,[25] it is rather difficult to assert the formation of these structures in Boc–Phe–Pro–Asn–Ala–Thr–NHMe. The fragment peptide analogues Boc–Pro–Asn–Ala–Thr–NHMe and Boc–Asn–Ala–Thr–NHMe show disordered structures but it is possible that Boc–Phe–Pro–Asn–Ala–Thr–NHMe takes an ordered structure in aqueous

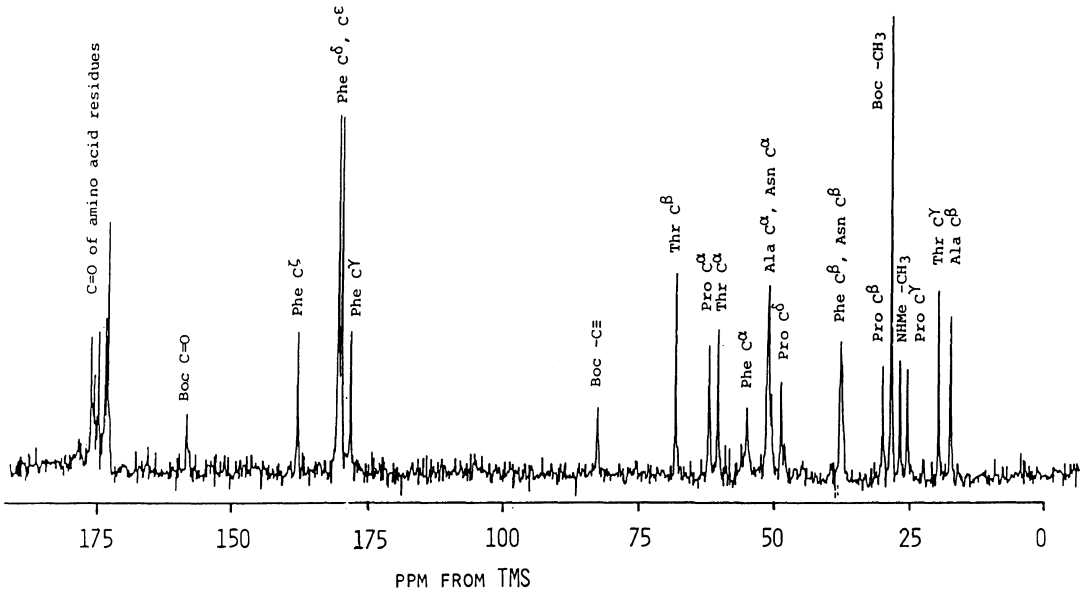

Figure 2. ^{13}C-NMR spectrum of Boc–Phe–Pro–Asn–Ala–Thr–NHMe in ^2H_2O solution at p^{2}H 7.

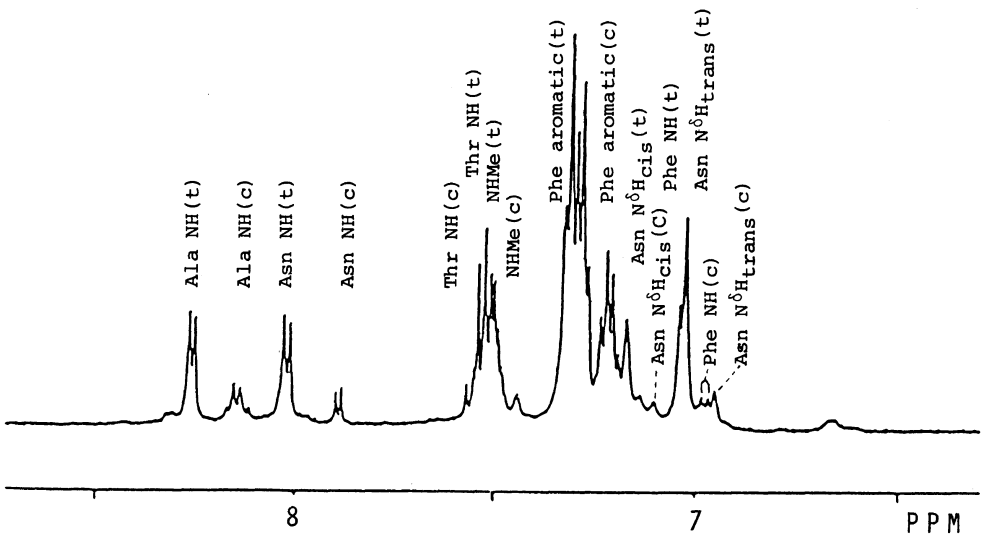

Figure 3. ^{1}H NMR spectrum of amide and aromatic proton region for Boc–Phe–Pro–Asn–Ala–Thr–NHMe in DMSO-d_6. (*c*) and (*t*) denote *cis* and *trans* isomer, respectively.

Table I. Chemical shifts (δ) of the ^{13}C NMR signals of Pro in Boc–Phe–Pro–Asn–Ala–Thr–NHMe measured in $^{2}H_2O$ solution (0.2 M) at 305 K

C^{α}	C^{β}	C^{γ}	C^{δ}	$\Delta\delta_{\beta\gamma}$[a]
62.03	30.33	25.87	49.02	4.46

[a] Chemical shift difference between C^{β} and C^{γ}.

solution. What type of structure it takes cannot be determined from CD data but formation of the ordered structure is dependent on peptide chain length. The results obtained here are consistent with previous data.[7]

The ^{13}C NMR spectrum of Boc–Phe–Pro–Asn–Ala–Thr–NHMe in aqueous solution is shown in Figure 2. The resonances were assigned by comparison with the literature.[26] The spectrum indicates that one conformer around the Phe–Pro bond is predominant in solution, the preferred conformer being *trans* from examination of the ^{13}C chemical shift of the Pro residue (Table I).[27] This is in good agreement with the finding that the *trans* isomer is predominant for Pro-containing peptides in aqueous solution.[28] The chemical shift differences between C^{β} and C^{γ}, $\Delta\delta_{\beta\gamma}$, can provide information on the probability of a particular peptide conformation, *i.e.*, it can be used to estimate the occurrence of a γ-turn structure.[25] The value thus obtained was 4.46, indicating a probability of approximately 0.25, suggesting that a turn structure is less likely in aqueous solution for Boc–Phe–Pro–Asn–Ala–Thr–NHMe.

The 500 MHz proton spectrum of Boc–Phe–Pro–Asn–Ala–Thr–NHMe was assigned by selective homo-spin decoupling and a comparison with its related fragment analogues. The amide/aromatic region (Figure 3) also shows isomerization around the Phe–Pro bond.

In Table II, the two isomers are present in solution and their chemical shift differences for any given proton decrease with increasing distance from the Phe–Pro bond. The *cis*/*trans* ratio of Boc–Phe–Pro–Asn–Ala–Thr–NHMe, calculated from the relative peak intensities of each set of amide proton resonances, is about 1/3. A similar value of 22/78 was reported for Ala–Phe–Pro–Ala in DMSO-d_6 solution.[29]

Temperature dependence studies in both aqueous and DMSO-d_6 solutions are tabulated

Table II. Chemical shifts (ppm) and vicinal proton coupling constants $^3J(HNC^{\alpha}H)$ (Hz) of amide protons for Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragments in DMSO-d_6 solution (0.03 M) at 298 K

	Isomer	Phe $N^{\alpha}H$		Asn $N^{\alpha}H$		Ala $N^{\alpha}H$		Thr $N^{\alpha}H$		NHMe	Asn $N^{\delta}H$ (*trans*)	Asn $N^{\delta}H$ (*cis*)
		δ	^3J	δ	^3J	δ	^3J	δ	^3J	δ	δ	δ
Boc–Phe–Pro–Asn–Ala–Thr–NHMe	*trans*	7.017	7.95	8.008	7.95	8.243	6.70	7.519	9.15	7.490	7.157	7.009
	cis	6.961	8.55	7.879	6.70	8.131	7.30	7.547	9.15	7.472	7.097	6.935
Boc–Pro–Asn–Ala–Thr–NHMe	*trans*			7.928	n.d.[a]	8.224	6.25	7.602	7.60	7.543	7.252	7.132
Boc–Asn–Ala–Thr–NHMe				6.870	8.55	8.035	6.70	7.637	8.55	7.524	7.336	6.879
Boc–Ala–Thr–NHMe						7.227	7.08	7.348	8.30	7.556		

[a] Not determined.

Table III. Temperature dependence, $d\delta/dT$, of the amide proton chemical shifts for Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragments measured in a mixture of H_2O–^2H_2O (9:1, v/v) at 0.03 M

	$d\delta/dT/10^3$ ppm deg^{-1}						
	Phe $N^{\alpha}H$	Asn $N^{\alpha}H$	Ala $N^{\alpha}H$	Thr $N^{\alpha}H$	NHMe	Asn $N^{\delta}H$ (*trans*)	Asn $N^{\delta}H$ (*cis*)
Boc–Phe–Pro–Asn–Ala–Thr–NHMe[a]	−7.15	−6.20	−5.95	−5.36	−4.36	−4.25	−3.95
Boc–Pro–Asn–Ala–Thr–NHMe[a]		−6.35	−8.91	−6.04	−6.35	−5.98	−4.64
Boc–Asn–Ala–Thr–NHMe		−8.53	−7.75	−7.83	−6.17	−5.59	−5.22
Boc–Ala–Thr–NHMe			−8.95	−8.08	−6.45		

[a] *trans* Isomer.

Table IV. Temperature dependence, $d\delta/dT$, of the amide proton chemical shifts for Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragments measured in DMSO-d_6 solutions at concentration of 0.03 M

		$d\delta/dT/10^3$ ppm deg^{-1}				
	Isomer	Phe $N^{\alpha}H$	Asn $N^{\alpha}H$	Ala $N^{\alpha}H$	Thr $N^{\alpha}H$	NHMe
Boc–Phe–Pro–Asn–Ala–Thr–NHMe	*trans*	−9.07	−3.40	−4.23	−3.18	−3.73
	cis	−7.16	−2.97	−4.00	−3.50	−3.60
Boc–Pro–Asn–Ala–Thr–NHMe	*trans*		−5.15	−4.80	−6.61	−5.09
Boc–Asn–Ala–Thr–NHMe			−4.38	−3.03	−2.80	−3.64
Boc–Ala–Thr–NHMe				−8.46	−2.08	−4.62

in Table III and IV, respectively. The chemical shift change of the carboxyamide protons of the Asn side chain is almost linearly dependent on the reciprocal of temperature change in aqueous solution, while there is no linearity in the DMSO-d_6 solution. Although the temperature dependent shift in aqueous solution for each amide proton in Boc–Phe–Pro–Asn–Ala–Thr–NHMe is larger than that in the fragment peptides, all $d\delta/dT$ values are smaller than the threshold value for hydrogen bonded amide protons (-3.00×10^{-3} ppm deg^{-1}),[30] indicating that there are no stable intramolecular hydrogen bonds in Boc–Phe–Pro–Asn–Ala–Thr–NHMe. Data obtained in DMSO-d_6 solution are similar. Although amide protons show larger values of $d\delta/dT$ compared to amides in aqueous solution, they are still below the threshold value for intramolecular hydrogen bonding.

However, the amide proton of Thr showed larger values of $d\delta/dT$ than those proposed for hydrogen bonded amides[30] in Boc–Asn–Ala–Thr–NHMe and more so in Boc–Ala–Thr–NHMe. Taking into account the short chain lengths of the latter peptides, this $d\delta/dT$ value may be attributed to an intramolecular hydrogen bond between the amide and side chains of Thr.

Although the formation of an ordered structure was suggested from CD data of Boc–Phe–Pro–Asn–Ala–Thr–NHMe, amide dependence studies in both aqueous and DMSO-d_6 solutions do not support such a conclusion. The preferred conformation for this peptide does not seem to be either a β-turn structure with a Pro–Asn corner or a γ-turn structure around the Pro residue.

Table V. NMR parameters of Thr for Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragments measured in DMSO-d_6 solution (0.03 M) at 298 K

	$^3J(HC^\alpha C^\beta H)$[a]	P_{g^-}[b]	$P_t+P_{g^+}$[b]	δOH[c]	$d\delta_{OH}/dT$[e]	$J(HC^\beta OH)$[a]
Boc–Phe–Pro–Asn–Ala–Thr–NHMe[f]	3.65	0.10	0.90	4.751	−4.46	5.45
Boc–Pro–Asn–Ala–Thr–NHMe[f]	3.60	0.09	0.91	4.790	−4.01	5.60
Boc–Asn–Ala–Thr–NHMe	3.50	0.08	0.92	4.783	−3.93	5.20
Boc–Ala–Thr–NHMe	3.30	0.06	0.94	4.933	−5.81	5.28

[a] Coupling constants in Hertz.
[b] Rotamer populations calculated using Pachler's approximation.
[c] Chemical shifts of the hydroxyl protons in ppm.
[d] In ppm deg.
[e] In 10^3 ppm deg^{-1}.
[f] *trans* Isomer.

As tabulated in Table II, there are differences in the values of vicinal coupling constant, $^3J(HNC^\alpha H)$, of Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragment peptides. Therefore, the possibility of side chain-side chain interactions was also studied. The NMR parameters for Thr residues in DMSO-d_6 solution are shown in Table V. The values for coupling constants $^3J(HC^\alpha C^\beta H)$ and $^3J(HC^\beta C^\gamma H)$ are similar for all peptides within experimental error, indicating almost equivalent conformations around the C^α–C^β and C^β–C^γ bonds throughout the series. The relative populations of the three stable rotamers calculated by using Pachler's approximation,[31] showed the (g^-)-rotamer (Figure 4) around the C^α–C^β bond to be sparsely populated for all Thr residues.

A temperature dependence study of the hydroxyl proton chemical shifts of the analogues showed that values of $d\delta/dT$ of peptides containing the triplet sequence were larger than in Boc–Ala–Thr–NHMe. Also, as the chemical shift of the hydroxyl proton in Boc–Ala–Thr–NHMe is different from those in Boc–Phe–Pro–Asn–Ala–Thr–NHMe, Boc–Pro–Asn–Ala–Thr–NHMe and Boc–Asn–Ala–Thr–NHMe, shielding of the hydroxyl protons is suggested on formation of the triplet sequence, though it is rather difficult to compare $d\delta/dT$ values for peptides of various chain lengths. Therefore, the possibility of a weak intramolecular interaction cannot be completely excluded for peptides containing the triplet sequence.

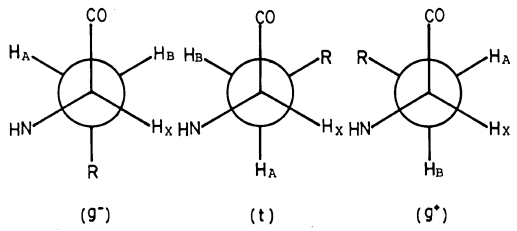

Figure 4. Three stable rotamers about the C^α–C^β bond. Values of dihedral angle χ^1 are −60°, 180°, and 60° for (g^-)-, (t)- and (g^+)-rotamers, respectively.

The side chain rotamer populations for Phe and Asn residues are also tabulated in Table VI. The predominance of the (g^-)-rotamer in Phe is a general characteristic of an amino acid with a bulky side chain in polar solvents.[32] In the case of Asn, although no populations for Asn containing analogues could be determined due to spectral overlap, the (g^-)-rotamer seemed to be the most populated. This is consistent with data from Asn-containing peptides[33] but inconsistent with those for Boc–Asn–Gly–Ser–OMe.[5] The small $d\delta/dT$ values for the carboxyamide protons of the Asn residues in aqueous solution (Table III) also indicate these protons do not participate in stable intramolecular hydrogen bonding. However, larger $d\delta/dT$ values with increasing chain length of the analogues reflect increased

Table VI. Vicinal coupling constants $^3J(HC^{\alpha}C^{\beta}H)$ (Hz) and rotamer populations[a] of Phe and Asn for Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragments in DMSO-d_6 solutions (0.03 M) at 298 K

	Phe					Asn				
	J_{AX}	J_{BX}	P_{g^-}	P_t	P_{g^+}	J_{AX}	J_{BX}	P_{g^-}	P_t	P_{g^+}
Boc–Phe–Pro–Asn–Ala–Thr–NHMe	10.40	4.47	0.71	0.17	0.12	n.d.[b]	6.42		0.35	
Boc–Pro–Asn–Ala–Thr–NHMe						7.55	5.50	0.45	0.26	0.19
Boc–Asn–Ala–Thr–NHMe						7.26	n.d.[b]	0.45		

[a] The population values were calculated by Pachler's approximation.
[b] Not determined.

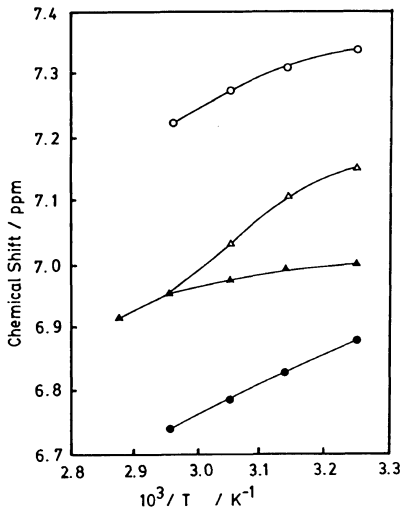

Figure 5. Temperature dependence of carboxyamide proton chemical shifts of Asn in DMSO-d_6 solutions. (○) and (●) denote *trans* and *cis* carboxyamide protons in Boc–Asn–Ala–Thr–NHMe. (△) and (▲) denote *trans* and *cis* those in Boc–Phe–Pro–Asn–Ala–Thr–NHMe.

shielding of the carboxyamide protons. In the temperature dependence studies of Asn carboxyamide protons in Boc–Phe–Pro–Asn–Ala–Thr–NHMe in DMSO-d_6 solution, the *cis* and *trans* carboxyamide protons, unlike in Boc–Asn–Ala–Thr–NHMe, collapsed to a single resonance at about 65°C (Figure 5). This collapse is due to the effects of internal rotation about the C^{β}–N^{γ} bond. Therefore, it is rather difficult to estimate the hydrogen bonding ability from temperature dependent shifts. But it can be said that the initial slope of the temperature dependent shift for Boc–Phe–Pro–Asn–Ala–Thr–NHMe is similar to both *cis* and *trans* carboxyamide protons in Boc–Asn–Ala–Thr–NHMe. Also, the *cis* temperature dependent slope is much smaller than that of the *trans* slope. In addition, chemical shift differences between the *cis* and *trans* carboxyamide protons were 0.148 and 0.457 ppm for Boc–Phe–Pro–Asn–Ala–Thr–NHMe and Boc–Asn–Ala–Thr–NHMe, respectively, and a value of 0.39 ppm was observed for Boc–Asn–NHMe.[33] These results indicate that the hydrogen bonding tendency of the carboxyamide protons of the Asn side chain in Boc–Phe–Pro–Asn–Ala–Thr–NHMe may differ from that in Boc–Asn–Ala–Thr–NHMe and also Boc–Asn–NHMe.

From the NMR study of the Asn and Thr side chains, it is concluded that the hydrogen bonding ability of the Thr side chain is affected by formation of the triplet sequence, *i.e.*, the hydroxyl protons of the Thr in the triplet sequence are shielded more than that in Boc–Ala–Thr–NHMe. Also, the intramolecular environment of the Asn side chain could be affected by backbone conformation in Boc–Phe–Pro–Asn–Ala–Thr–NHMe. Since the temperature dependent shifts become smaller on formation of the triplet sequence not only for the hydroxyl proton but also for the Asn carboxyamide proton, it seems unlikely that there is only one stable hydrogen bond between Asn and Thr side chains in the triplet

sequence.

In the flexible peptides investigated, the NMR data only indicate possible weak intramolecular interactions between Asn and Thr side chains. Although CD data for Boc–Phe–Pro–Asn–Ala–Thr–NHMe support the possibility of a loop structure such as a β or γ-turn, the presence of some stable secondary structure is unlikely. A peptide containing the triplet sequence may have the potential to form some kind of loop structure rather than be already located in one. Such a potential may be realized in the *N*-glycosyltransferase membrane environment where a loop structure may form to facilitate glycosylation of the Thr side chain.

CONCLUSION

From the CD and NMR studies of Boc–Phe–Pro–Asn–Ala–Thr–NHMe and its fragment analogues, the following conclusions are drawn. The potential for some ordered structure increases with increasing chain length of the analogues but there is no stable intramolecular hydrogen bonds involving backbone amide protons. The triplet sequence itself is not associated with any specific secondary structures. But changes in the hydrogen bonding potentials of Thr hydroxyl protons on inclusion in the triplet sequence and dependence of the carboxyamide protons of Asn residues on backbone conformations indicate the importance of the ability to form intramolecular hydrogen bonds between Asn and Thr side chains.

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