

Localisation and Importance of the Sugar Part of Human Casein

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The study of glycopeptides obtained after enzymic digestions of human caseinoglycopeptide demonstrated the presence of several (up to 10) prosthetic sugar groups distributed throughout the peptide chain instead of only one in the case of cow and sheep κ -caseinoglycopeptides. One of the glycopeptides allowed us to complete the C-terminal sequence of human caseinoglycopeptide previously published in part by others. The predicted secondary structure was in accordance with the high number of sugar groups situated all but one in β -turns. A significant microheterogeneity seems to occur at the sugar level of human caseinoglycopeptide, which might thus present differences from one milk to another.

κ -Casein is the main glycoprotein of cow milk. It is the principal casein fraction affected by chymosin and is also the only casein fraction that contains sugars [1–4]. During the primary phase of chymosin action, Jollès demonstrated that a Phe $\rightarrow$ Met linkage is specifically split [5]; an insoluble part, para- κ -casein, is formed and a soluble fraction is released which is called caseinoglycopeptide as it contained all the sugars of cow κ -casein (around 10% in the peptide). Only three monosaccharide constituents have been identified, galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic acid [6]. However, the establishment of their sequence was not easy because of microheterogeneity at the sugar level [7]. By enzymic digestions a single glycopeptide was isolated from κ -casein, threonine residue 133 was identified as the single sugar-bearing amino acid and the linkage was characterized as *O*-glycosidic [8]. The sequences of the three main carbohydrate portions were established by mass spectrometry or nuclear magnetic resonance [9, 10].

Human κ -casein has not yet been entirely purified; however, from its chymosin digest the pure caseinoglycopeptide was obtained; it contains approximately five times more sugars (around 55%) than cow caseinoglycopeptide [11]. Not only galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic acid, but also fucose and *N*-acetylglucosamine were identified. It was thus tempting to suggest the presence of more

than one polysaccharide attachment site. The present paper deals with the primary and secondary structures of human caseinoglycopeptide as well as with the purification and the localisation of several glycopeptides isolated from different areas of the caseinoglycopeptide in order to clarify the question of the number of prosthetic sugar groups.

MATERIALS AND METHODS

Materials

Sephadex and DEAE-Sephadex A-25 were obtained from Pharmacia and Dowex 50X2 from Bio-Rad. Carboxypeptidase A and trypsin were purchased from Worthington, neuraminidase from *Cl. perfringens* from Sigma and pronase P from *Str. griseus* from California Corporation for Biochemical Research. All other reagents (analytical grade) were purchased from Merck or Prolabo, except those employed for the sequencer which were obtained from S.D.S. (Marseille).

Preparation of Human Casein and Caseinoglycopeptide

Whole human casein from fresh pooled milk was prepared according to Alais and Jollès [12]. Chymosin (enzyme/substrate: 1/2000) was added to a 2% solution of whole casein in a 0.2 M pyridine/acetic acid buffer, pH 5.5. After digestion during 1.5 h at 37 °C, trichloroacetic acid (final concentration 12%) was added; the supernatant contained the caseinoglycopeptide [11]. Further purification included several washings of the

This paper is the 44th communication on caseins.

Enzymes. Carboxypeptidase A (EC 3.4.17.1), trypsin (EC 3.4.21.4), neuraminidase (EC 3.2.1.18), pronase P (EC 3.4.24.4), chymosin (EC 3.4.23.4).

supernatant with diethyl ether, dialysis against distilled water (4°C), concentration with an Amicon concentrator (UM 2000), filtration on Sephadex G-25 (25 × 4 cm) with 10 mM NH₄OH as eluent, chromatography on DEAE-Sephadex A-25 (30 × 3 cm) equilibrated with a 0.03 M Tris-HCl buffer, pH 7.8 followed by a gradient elution (addition of 0.5 M NaCl) and a final filtration on Sephadex G-25 (200 × 2 cm) with 30% acetic acid as eluent. The purity of the caseinoglycopeptide was ascertained by automated Edman degradation. From 6 g whole casein, 35–40 mg caseinoglycopeptide was obtained after the purification.

Preparation of a Long Tryptic Glycopeptide from Human Caseinoglycopeptide

The tryptic peptides of the caseinoglycopeptide were obtained after enzymic digestion (enzyme/substrate: 1/100; 0.1 M ammonium bicarbonate, pH 7.9) during 24 h at 37°C. Trypsin was pretreated during 16 h with 62.5 mM HCl at 37°C. The enzymic digest was filtered on Sephadex G-25 (150 × 1.2 cm) with 30% acetic acid as eluent and two peaks were characterized: the first contained a long fragment with all the sugars and the second a mixture of shorter tryptic peptides.

Preparation of Short Glycopeptides Obtained by Pronase Digestion from Human Caseinoglycopeptide

Human caseinoglycopeptide (71 mg) was digested successively with neuraminidase, trypsin and pronase. Digestion with neuraminidase (enzyme/substrate ratio: 1/1290, by weight) was achieved in 0.1 M sodium acetate containing 1 mM calcium chloride at pH 5.0, 37°C, during 24 h. After dialysis and concentration (Amicon UM 2000) digestion with pretreated trypsin was carried out in a 0.025% trimethylamine solution (pH 8.0) at 37°C during 8 days (enzyme/substrate ratio: 1/50). Digestion with pronase P was achieved in a 0.025% trimethylamine solution, pH 8.0, containing 1 mM calcium chloride. After addition of the enzyme the digestion was continued during 8 days at 37°C (enzyme/substrate ratio: 1/120) in the presence of a drop of toluene. The final digest was filtered on Sephadex G-25 (200 × 2 cm; 30% acetic acid as eluent): a single broad sugar-rich fraction was detected. It was chromatographed on DEAE-Sephadex A-25 (22 × 1.3 cm) equilibrated with a 50 mM pyridine/acetic acid buffer, pH 5, as described in the legend to Fig. 1.

Analytical Procedures

Amino acid analyses were performed after total hydrolysis (6 M HCl; 18 h; under vacuum) with a

Technicon amino acid Autoanalyzer. The peptides were characterized by ninhydrin after alkaline hydrolysis. The amino sugars were determined after hydrolysis (4 M HCl; 4 h; 110°C) according to Rondle and Morgan [13] and also with the amino acid Autoanalyzer. Phosphoserine was detected after hydrolysis (10.5 M HCl; 8 h; 80°C) with the Autoanalyzer.

Structural Methods

N-terminal amino acids were identified by the dansylation technique [14]. C-terminal amino acids were released by digestion with carboxypeptidases A (37°C; 0.1 M sodium bicarbonate) during different time intervals and were identified with the Autoanalyzer. Automated Edman degradation [15] was carried out in a Beckman Sequencer Model 890 C using a 1 M quadrol [*N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine] program for the long tryptic glycopeptide and a 0.1 M quadrol program in the presence of polybrene [16] for the short glycopeptides modified according to Foster et al. [17]. The thiazolinones were converted into phenylthiohydantoin amino acids and the latter were characterized by thin-layer chromatography or gas liquid chromatography or high-performance pressure liquid chromatography.

Alkali-sensitive *O*-glycosidic linkages in human caseinoglycopeptide were characterized according to the procedure previously employed in the case of cow caseinoglycopeptide [18].

Prediction of Secondary Structure

The predictive rules formulated by Chou and Fasman [19] were applied to predict the secondary structure. The assignment of residues in terms of helical or β -sheet potentials was obtained using the parameters given by Fasman et al. [20,21]. The relative probability that a tetrapeptide forms a β -turn is $p_i = f_i \cdot f_{i+1} \cdot f_{i+2} \cdot f_{i+3}$ [22]. The frequencies of occurrence, $f_i, f_{i+1}, f_{i+2}, f_{i+3}$ of a given residue in the first, second, third and fourth positions, respectively, of a β -turn, are those indicated by Chou and Fasman [23]; these values are based on the study of 408 β -turns. The averaged value of p_i for a known β -turn was thus found to be 0.5×10^{-4} [23]. In the case of human caseinoglycopeptide the averaged value of p_i is 0.63×10^{-4} . This averaged figure might result in over-prediction and the use of a somewhat higher (1.5 times) value was suggested [24]. Therefore the minimum value of the probability for a given tetrapeptide to adopt the β -turn conformation should be $p_i = 0.94 \times 10^{-4}$. In the present research we shall discuss the probability of existence of a β -turn taking both values (0.94×10^{-4} and 0.5×10^{-4}) as the cut-off values. In addition if $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$ are average conformational param-

T = tryptic peptide. 1a, 1b, 2 = peptides from pronase digest. →, sequence determined by automated Edman degradation; —, amino acid released by carboxypeptidase A digestion

1 Ile-Ala-Ile-Pro-Pro-Lys-Lys-Ile-Gln-Asp-Lys-Ile-Ile-Ile-Pro-Thr-Ile-Asn-
Caseinoglycopeptide
Tryptic glycopeptide
Pronase glycopeptide

19 Thr-Ile-Ala-Thr-Val-Glu-Pro-Thr-Pro-Ala-Pro-Ala-Thr-Glu-Pro-Thr-Val-Asp-
Caseinoglycopeptide
Tryptic glycopeptide
Pronase glycopeptide

37 Ser-Val-Val-Thr-Pro-Glu-Ala-Phr-Thr-Glu-Ser-Ile-Ile-Thr-Ser-Thr-Pro-Glu-
Tryptic glycopeptide
Pronase glycopeptide

55 Thr-Pro-Thr-Val-Ala-Val-Pro-Thr-Thr-Ser-Ala
Tryptic glycopeptide
Pronase glycopeptide
Caseinoglycopeptide

peptides of other origins, no phosphoserine or phosphothreonine was detected.

A main difficulty of the present study has been to determine the amino acid sequence of the peptide parts containing several prosthetic sugar groups. In sequencing the glycopeptide regions, Edman degradation proceeds normally through *O*-glycosylated amino acid residues, although frequently only with low yields [26]; however, the phenylthiohydantoin of glycosylated amino acid residues cannot be identified by conventional methods [26]. Only *O*-glycosylated sugar $\rightarrow$ peptide bonds have so far been characterized in α -caseins [8]; the presence of *N*-glycosylated linkages could be ruled out as the code sequence Asn-X-Ser/Thr did not occur in human caseinoglycopeptide. All the serine residues could be easily characterized: thus this amino acid was not involved in *O*-glycosidic linkages. When only one amino acid residue in a peptide is *O*-glycosylated, as in the present case (threonine), that residue can be determined from the amino acid composition as previously discussed by others [26, 27].

Strategy of the Sequence Determination of Human Caseinoglycopeptide. The Problem of the O-Glycosylated Amino Acid Residues

Structure of the Long Tryptic Glycopeptide

The long glycopeptide obtained after tryptic digestion of human caseinoglycopeptide constituted its C-terminal moiety (residues 12–65) as determined by amino acid analysis (Table 2). Residues 12–55 were directly sequenced (Table 1). No amino acid could be detected in positions 16, 26, 31, 34, 40, 45, 50 and 52; only traces of threonine were found in position 22. From the amino acid and amino sugar analyses it was obvious that *O*-glycosidic threonine residues occurred in the former positions.

Table 2. Amino acid and amino sugar compositions of human caseinoglycopeptide and of some glycopeptides obtained after enzymatic digestion
Nearest integer in parentheses

Amino acid or amino sugar	Human caseinoglyco- peptide (values inferred from the sequence)	Tryptic C-terminal glycopeptide	Glycopeptides after pronase digestion		
			1a	1b	2
residues/molecule					
Asp	3	2.4 (2)			1.0 (1)
Thr	14	14.0 (14)	6.3 (6+1) ^b	1.0 (1)	4.0 (4)
Ser	4	4.2 (4)	2.2 (2+1) ^b	—	1.0 (1)
Glu	6	5.0 (5)	1.4 (1+1) ^b	—	2.1 (2)
Pro	11	8.7 (9)	3.0 (3)	1.0 (1)	3.8 (4)
Ala	7	5.6 (6)	1.8 (2)	—	2.6 (2+1) ^c
Val	6	4.7 (5)	2.1 (2)	—	2.0 (2)
Ile	10	4.8 (8) ^a	1.1 (1 + 1) ^{a, b}	0.8 (1)	—
Phe	1	1.0 (1)	—	—	—
Lys	3	—	—	—	—
Glucosamine	17	15.4	6.5	1.3	6.5
Galactosamine	10	9.0	4.3	1	4.3

^a Extrapolated value after 72 h hydrolysis.

^b Fraction 1a is constituted by a mixture of two glycopeptides (see text) containing residues 45–65 (40%) and residues 49–65 (60%) respectively.

^c Fraction 2 is constituted by a mixture of two glycopeptides differing only by their N-terminal residue (see text).

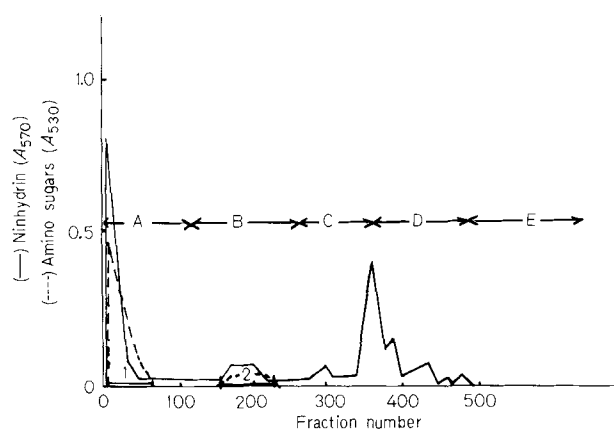


Fig. 1. Chromatography on DEAE-Sephadex A-25 (22 × 1.3 cm) of the sugar-rich fraction obtained after enzymic digestions and filtration on Sephadex G-25 of the human caseinoglycopeptide. (A) 50 mM pyridine/acetic acid buffer, pH 5.0; (B) gradient elution; addition of a 0.5 M pyridine/acetic acid buffer, pH 5.0, into a mixing chamber of 200 ml capacity containing buffer A; (C) gradient elution; addition of a 0.65 M pyridine/acetic acid buffer, pH 5.0; (D) 0.65 M pyridine/acetic acid buffer, pH 5.0; (E) 0.25 M NaOH. 2-ml fractions were collected

Purification and Sequence of Short Glycopeptides Obtained after Pronase Digestion

In order to corroborate the presence of more than one prosthetic sugar group per molecule we isolated short glycopeptides after enzymic (neuraminidase, trypsin and pronase) digestions of the caseinoglycopeptide. Their separation was achieved on DEAE-Sephadex A-25 during a gradient elution as indicated in Fig. 1: two sugar-rich peaks (1 and 2) were thus

obtained. Each of them was filtered on a Sephadex G-25 column (200 × 2 cm) with 30% acetic acid as eluent: peak 1 gave rise to two sugar-rich fractions (1a and 1b) and peak 2 to one sugar-rich fraction. Their amino acid and amino sugar compositions are reported in Table 2.

The sequence determination of these highly glycosylated peptides constituted again a major difficulty as it was not always possible to purify smaller glycopeptides to homogeneity, as previously noted by others [26].

The sugar-rich fraction 1a was automatically sequenced; it comprised two glycopeptides arising from the C-terminal moiety of the caseinoglycopeptide, Thr-45 → Ala-65 and Ile-49 → Ala-65, respectively; the ratio between both peptides was 30:70, according to the amino acid composition reported in Table 2. They were sequenced from residue number 45 up to residue 63 (Table 1). Threonine residue 45 was characterized by the dansylation technique.

Two of the threonine residues (55 and 57) were directly determined during automated Edman degradation. On the other hand no amino acid was detected in positions 45, 50, 52, 62 and 63; according to the amino acid and amino sugar analyses (Table 2), it was obvious that O-glycosidic threonine residues occurred in these positions. These data corroborated the results obtained with the long C-terminal tryptic glycopeptide.

The sequence of fraction 1b was easily established by classical methods (dansylation, amino acid analysis); it corresponded to the tripeptide; Ile-Pro-Thr with one residue of N-acetylgalactosamine. According

to Table 1, the localisation of peptide 1b was only possible between residues 14 and 16 of human caseinoglycopeptide.

The sugar-rich fraction 2 was constituted by two glycopeptides; they could not be easily separated as they arose from the same area of human caseinoglycopeptide and differed only by their N-terminal amino acid, characterized by the dansylation technique: Ala-21 → Ser-37 and Thr-22 → Ser-37; the ratio between both peptides was 66:33. No threonine residue could be directly identified during automated Edman degradation in positions 26, 31 and 34; thus we assume that they are glycosylated, more especially as several residues of galactosamine were characterized in fraction 2 (Table 2). In position 22, traces of threonine were detected.

In conclusion, the sequences of the glycopeptides isolated from fractions 1a, 1b and 2 were in accordance with the structure indicated in Table 1.

C-Terminal Sequence

Sequence studies carried out with glycopeptide 1a allowed us to establish the partial C-terminal sequence: ... Val-Pro-(Thr, Thr, Ser, Ala) (see Table 1). After digestion of the caseinoglycopeptide with carboxypeptidase A (24 h), serine and alanine were obtained with a yield of 23% and 44% respectively, establishing the C-terminal sequence —Ser-Ala. In our experimental conditions threonine was not liberated; this result was in favour of the occurrence of *O*-glycosylated threonine residues in position 62 and/or 63.

Localisation of the *O*-glycosylated Threonine Residues

Among the 14 threonine residues of human caseinoglycopeptide, three (numbers 19, 55 and 57) could be directly determined during Edman degradation. Only traces could be detected in position 22. No amino acid was characterized by the same technique in positions 16, 26, 31, 34, 40, 45, 50, 52, 62 and 63. The latter ten threonine residues seem thus to be involved in *O*-glycosidic linkages.

These data were in accordance with experiments carried out in order to characterize directly the presence of *O*-glycosidic groups in human caseinoglycopeptide. When the latter was treated with 0.5 M NaOH, an increase of the absorbance at 241 nm as a function of time occurred; it was five times greater than the increase observed under similar experimental conditions with cow α -caseinoglycopeptide, which contained only a unique *O*-glycosidic sugar group. It is not possible to obtain by this procedure a more precise quantitative result, but it favours the presence of a higher number of prosthetic sugar groups in human than in cow caseinoglycopeptide.

Table 3. Conformational prediction of α -helix, β -sheet and β -turn regions in human caseinoglycopeptide

Conformation	Region	$\langle p_i \rangle \times 10^4$	$\langle P_T \rangle$	$\langle P_\alpha \rangle$	$\langle P_\beta \rangle$
α -helix	8 → 13 ^a			1.09	1.19
β -sheets	18 → 23			0.98	1.23
	57 → 60			1.09	1.35
β -turns	4 → 7	3.30	1.26	0.86	0.64
	14 → 17	1.18	0.85	0.89	1.2
	24 → 27	1.41	1.18	0.87	0.66
	26 → 29	0.95	1.65	0.85	0.78
	32 → 35	1.39	0.95	0.99	0.95
	40 → 43	2.05	0.95	0.87	0.73
	44 → 47	1.03	0.93	1.06	0.92
	49 → 52	0.87	0.97	0.87	1.18
	52 → 55	2.81	1.04	0.93	0.82
	61 → 64	1.26	1.22	0.75	0.92

^a Even if $\langle P_\beta \rangle > \langle P_\alpha \rangle$, this sequence is predicted as helical (see text).

Prediction of the Secondary Structure of Human Caseinoglycopeptide

α -Helical and β -Sheet Regions. The predicted α -helical and β -sheet regions are summarized in Table 3. Sequence 8 → 13 was predicted as helical although $\langle P_\beta \rangle > \langle P_\alpha \rangle$, because it contains two amino acids which are β -sheet breakers, Asp-10 and Lys-11, but no α -helix breaker. Moreover, this helical sequence is stabilized by the polar interaction between residues 10 and 11. The two β -sheet sequences, residues 18 → 23 and 57 → 60, are characterized by a markedly high value for $\langle P_\beta \rangle$ (1.23 and 1.35). The relative composition of the conformational regions are as follows: 9.2% α -helix (one region including six residues) and 15.4% β -sheet (two regions including a total of ten residues).

β -Turns. The computed β -turns profile with the two cut-off values (see Materials and Methods) is indicated in Fig. 2. 12 and 14 peaks are situated above the cut-off points 0.94×10^{-4} and 0.50×10^{-4} respectively. The localization of the threonine residues was important; indeed, as previously established [28–31], carbohydrate-peptide linkages were situated predominantly in β -turn regions. As noted above, three threonine residues (19, 55 and 57) could be directly determined during the Edman degradation and thus do not carry a carbohydrate moiety. Traces of threonine were found in position 22. Threonine residues 16, 26, 31, 34, 40, 45, 50, 52, 62 and 63 were not detected during Edman degradation and seem thus to be glycosylated. Fig. 2 indicates that nearly all these residues were situated in a β -turn tetrapeptide whatever the cut-off value. This is the case for residues 16, 26, 34, 40, 45, 52, 62 and 63. Among the two other threonines, residue 31 was located in the vicinity of a β -turn and of a proline; residue 50 was located in a β -turn only when 0.5×10^{-4} was chosen as cut-off

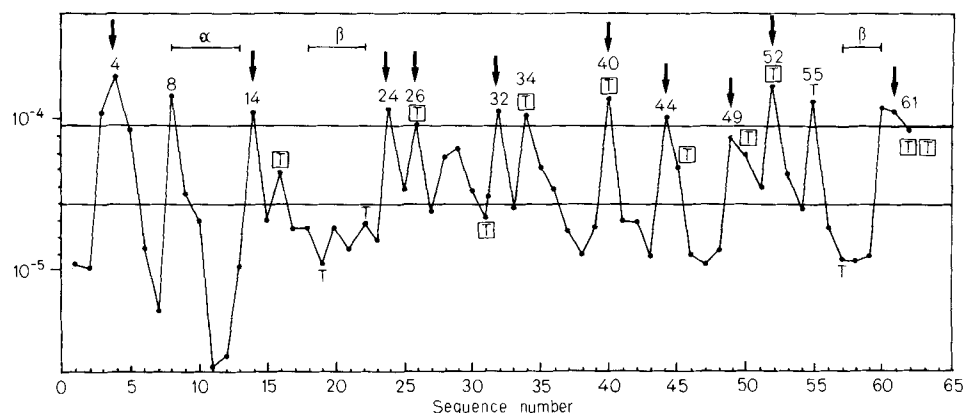


Fig. 2. Probability of tetrapeptide β -turns in human caseinoglycopeptide. The horizontal lines correspond to two cut-off values of 0.94×10^{-4} and 0.5×10^{-4} (see Materials and Methods). The 10 β -turns predicted in the secondary structure, with a cut-off value of 0.5×10^{-4} , are indicated by an arrow. The 14 threonines of the caseinoglycopeptide are indicated by T, either in a square box, corresponding to the O-glycosylated residues, or without a box, corresponding to the unglycosylated residues. The α -helical and β -sheet regions determined in Table 1 are also shown

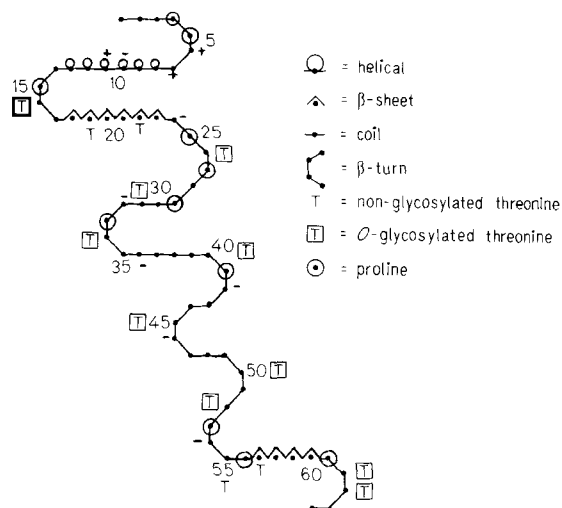


Fig. 3. Schematic diagram of the predicted secondary structure of human caseinoglycopeptide. Charged residues (+, -) are indicated on the diagram

value. Residue 22 never did occur in a β -turn whatever the cut-off value; as only traces of threonine were detected, this residue might be particularly labile. The general characteristics of the β -turn regions of human caseinoglycopeptide are presented in Table 3. The sequences $14 \rightarrow 17$, $32 \rightarrow 35$, $44 \rightarrow 47$, $49 \rightarrow 52$ and $52 \rightarrow 55$ are included although their conformational parameters $\langle P_T \rangle$ are slightly below the $\langle P_\alpha \rangle$ and $\langle P_\beta \rangle$ values as their p_i values are markedly higher than the cut-off value. Moreover, it is well known that the folding of the chain as a β -turn occurs more preferentially when a proline residue is located in the second position [23] of the β -turn: this is the case for the tetrapeptides $14 \rightarrow 17$, $32 \rightarrow 35$ and $52 \rightarrow 55$.

In conclusion ten β -turns corresponding to 57% of the molecule could be predicted with one overlap for the $24 \rightarrow 27$ and $26 \rightarrow 29$ turns. The complete secondary structure of the protein moiety of human caseinoglycopeptide is shown in a schematic diagram (Fig. 3). The α -helical (residues $8 \rightarrow 13$) and β -sheet (residues $18 \rightarrow 23$) domains separated by the predicted $14 \rightarrow 17$ β -turn seem to occur in an α -helical- β -sheet interaction.

DISCUSSION

The study of glycopeptides obtained after enzymic digestions of human caseinoglycopeptide from pooled milk allowed us [a] to complete the sequence of its C-terminal moiety and (b) to demonstrate the presence of several prosthetic sugar groups. This result establishes an important difference between cow or sheep and human caseinoglycopeptides as the former possess only a single sugar-bearing threonine [5] while the latter contains up to 10 O-glycosidic threonines. This figure is consistent with the predicted secondary structure.

Human caseinoglycopeptide presents a high percentage of organized structure (82%). 57% correspond to β -turns; this value is very high, especially in comparison to cow and sheep caseinoglycopeptides, which contain only 25% of β -turns [32]. This result is, however, in accordance with the high number of O-glycosidic threonine residues demonstrated during the Edman degradation, which occur all but one in β -turns. The question remains open whether threonine residues, which are not situated in a β -turn, can be glycosylated. Finally it is worth mentioning that charged residues are always located either in a β -turn tetrapeptide or in a region very near of a β -turn.

except for the charged residues 10 and 11 which stabilize the α -helical sequence 8 $\rightarrow$ 13 (Fig. 3).

In cow and sheep α -caseinoglycopeptides no *N*-acetylglucosamine was detected; in the human peptide this amino sugar must form a part of the *O*-glycosidic *N*-acetylgalactosamine-containing prosthetic groups as the absence of the code sequence Asn-X-Ser/Thr rules out the occurrence of *N*-glycosidic linkages. A preliminary series of investigations achieved with glycopeptide 1b, where threonine residue 16 was involved, demonstrated microheterogeneity at the sugar level as previously noted in the case of the prosthetic group of cow caseinoglycopeptide [9,10]. Threonine residue 55 should also be mentioned, as it is not glycosylated although situated in a β -turn. It is quite possible that in another milk sample this residue might bear a sugar group when another threonine might be devoid of such a group; the possibilities of microheterogeneity at the sugar level are thus increased. The possible existence of a specific caseinoglycopeptide for a woman or a group of women can only be settled by the study of individual milks, which at the present time is rather difficult because of the low yields of the glycopeptides obtained after enzymic digestions.

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