Conformational aspects of *N*-glycosylation of proteins

Studies with linear and cyclic peptides as probes

Ernst BAUSE, Harald HETTKAMP and Günter LEGLER

*Institut für Biochemie der Universität zu Köln, Zùlpicherstrasse 47, D-5000 Köln 1,
Federal Republic of Germany*

(Received 25 January 1982/Accepted 1 March 1982)

Conformational aspects of *N*-glycosylation of glycoproteins have been studied by using a series of peptides which contained, in addition to the 'marker sequence' Asn-Gly-Thr, two cysteine residues in various positions of the peptide chain. The presence of two cysteines permitted a partial fixation of the above triplet sequence in cyclic structures of various size by intramolecular disulphide bond formation. Comparison of the glycosyl acceptor properties of the linear peptides and their corresponding cyclic analogues allows the following statements. The considerably lower acceptor capabilities of the cyclic derivatives indicate that the restriction of rotational degrees of freedom imposed by disulphide bonding results in a conformation which hinders a favourable interaction of the peptide substrate with the *N*-glycosyltransferase. On the other hand, the glycosylation rate of linear peptides increases with increasing chain length, suggesting that the amino acids on both the *N*- and *C*-terminal side of the 'marker sequence' may contribute to a considerable extent to the induction of an 'active' conformation. Realization of a potential sugar attachment site requires a hydrogen bond interaction within the 'marker sequence' between the oxygen of threonine (serine) as the hydrogen bond acceptor and the β -amide of asparagine as the donor [Bause & Legler (1981) *Biochem. J.* **195**, 639–644]. This interaction is obviously facilitated when the peptide chain can adopt a conformation which resembles a β -turn or other loop structure. The available experimental and statistical data are discussed in terms of possible structural features for *N*-glycosylation, with the aid of space-filling models.

The biosynthesis of the *N*-glycosidic linkage in glycoproteins occurs in a post-translational process which involves a complex interplay among the nascent polypeptide chain being extruded through the membrane of the endoplasmic reticulum, the synthesis of a particular oligosaccharide on a polyisoprenyl diphosphate as lipophilic anchor group and its transfer *en bloc* onto an asparagine residue of the acceptor protein (for review, see Struck & Lennarz, 1980). Concerning the selection of an asparagine to function as sugar attachment site, it is suggested that the major determinants are encoded in the primary structure of the polypeptide chain itself.

First experimental support for the idea of a 'marker sequence' (Marshall, 1967; Neuberger &

Marshall, 1968) was obtained from studies in oviduct (Pless & Lennarz, 1977), thyroid (Ronin *et al.*, 1978), liver (Bause, 1979) and yeast (Bause & Lehle, 1979) which showed that exogenous peptides containing the triplet sequence Asn-Xaa-Thr could be glycosylated at asparagine when dolichyl diphosphate sugars were used as glycosyl donors. Recent work in our laboratory, carried out to study the functional role of the hydroxy amino acid in this 'marker sequence' for the process of glycosylation, has established that, besides its contribution to the formation of a recognition site, this amino acid might be directly involved in the catalytic mechanism of glycosyl transfer in that it is promoting the necessary proton transfer from the amide group of asparagine to a basic group of the *N*-glycosyltransferase (Bause & Legler, 1981).

The occurrence of the above triplet sequence, however, is obviously not the only parameter that determines to what extent a given site in the

Abbreviations used: Dol-*P*, dolichyl monophosphate; Dol-*PP*, dolichyl diphosphate; Xaa, 'unknown' or 'variable' amino acid.

polypeptide chain is glycosylated. In fact, it is found that only about one-third of known potential sugar attachment sites in eukaryotic proteins bear carbohydrate. Up to date, however, only little information is available about how additional determinants should look. The statistical studies of Aubert *et al.* (1976) and Beeley (1977) suggest that the process of sugar transfer onto the asparagine residue of the 'marker sequence' might be associated with the formation of specific secondary structures in the peptide chain. In order to explore this possibility, we have synthesized a series of model peptides which contained, in addition to the tripeptide sequence Asn-Gly-Thr, two cysteine residues in various positions of the peptide sequence. By means of the two cysteines, the linear peptides could be converted reversibly into their cyclic analogues by disulphide formation. We expected that alterations of the glycosyl-acceptor properties resulting from this modification might allow some statements about the conformation of the glycosyl acceptor site under conditions *in vivo*.

Materials and methods

Materials

The materials used and their sources were as follows: UDP-*N*-acetyl-[14 C]glucosamine (sp. radioactivity 323 Ci/mol), The Radiochemical Centre, Amersham; *t*-butoxycarbonyl amino acids, ninhydrin, dicyclohexylcarbodi-imide and dithiothreitol, Serva; dolichyl phosphate, UDP-*N*-acetylglucosamine and Triton X-100, Sigma; Bio Beads S-X1, chloromethylated, Bio-Gel P4, 200–400 mesh, Bio-Rad Laboratories; *t*-butoxycarbonyl-*S*-*t*-butylmercapto-L-cysteine, Fluka; silica-gel-coated glass plates and trifluoroacetic acid, Merck. All other chemicals were purchased from commercial sources in the highest purity available.

Preparation of calf liver microsomal membranes

Membranes were prepared in 50 mM-Tris/HCl buffer, pH 7.2, containing 0.25 M-sucrose by the use of a Potter homogenizer as described by Moulé *et al.* (1960). The homogenate was filtered through cheese-cloth and centrifuged at 10 000 *g* for 30 min. The supernatant was separated and recentrifuged at 48 000 *g* for 120 min. The resulting pellet was suspended in homogenizing buffer and used as the enzyme source. All operations were carried out at 4°C. The final enzyme preparation contained approx. 20 mg of protein/ml when assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

N-Glycosyltransferase assay and isolation of [14 C]-glycopeptides

The acceptor properties of the various peptides

were measured with Dol-PP-[14 C]chitobiose as the glycosyl donor and the above microsomal fraction of calf liver as enzyme source. Standard incubation mixtures contained, in a final volume of 100 μ l: $(2-10) \times 10^3$ c.p.m. of Dol-PP-[14 C]chitobiose, 50 mM-Tris/HCl, pH 7.2, 0.8% Triton X-100, 10 mM-MnCl₂, 100–200 μ g of microsomal protein and peptide as indicated. Acceptor capabilities of the reduced, linear peptide derivatives were measured after preincubation of the cyclic peptides in the presence of 10 mM-dithiothreitol for 20 min. Under these conditions complete reduction of disulphide bonds was achieved. The incubations were terminated by adding 1 ml of methanol to the incubation mixtures. The samples were centrifuged and the supernatants, which contained the water-soluble radiolabelled glycopeptides, were separated from the pellets and made biphasic by the addition of 2 ml of chloroform and 0.4 ml of water in order to extract small amounts of unreacted Dol-PP-[14 C]chitobiose. The upper aqueous phase was separated and counted for radioactivity. Controls which were run in parallel experiments in the absence of exogenous peptides showed that the water-soluble radioactivity, presumably originating from a partial hydrolysis of the [14 C]glycolipid, was generally below 5% of the total radioactivity used in the various experiments.

Synthesis of peptides

Peptides were prepared by the solid phase method as described by Merrifield (1963) and Erickson & Merrifield (1976), using chloromethylated Bio-Beads S-X1 as resin support. Amino acids were attached as their *t*-butoxycarbonyl derivatives by the dicyclohexylcarbodi-imide method, except for asparagine which was coupled as the *p*-nitrophenyl ester derivative. Side chain functions of threonine, serine and tyrosine were protected by benzylation. Cysteine was introduced as *t*-butoxycarbonyl-*S*-*p*-methoxybenzylcysteine or *t*-butoxycarbonyl-*S*-*t*-butylmercaptocysteine. Cleavage of peptides from the resin was done by treatment with anhydrous HF when the *S*-*p*-methoxybenzyl group was used as protecting group, or with HBr/trifluoroacetic acid in the presence of anisole as bromine scavenger in the case of *S*-*t*-butylmercaptyl protection. The latter protecting group was split off by reduction with dithiothreitol. The peptides were purified by gel chromatography on Bio-Gel P4 in the presence of 2 mM-dithiothreitol with 1.0 M-acetic acid as eluant.

Cyclization of reduced peptides

A diluted solution of the purified and reduced peptides was added dropwise and under pH control to a solution of 50 mM-NH₄HCO₃, pH 8.5, to give a final peptide concentration between 0.01 and 0.05 mg/ml of solution. Oxidation in this high

dilution favours cyclization over polymerization. After stirring for 24 h in an open beaker to allow access of oxygen, no free thiol groups were detectable with Ellman's reagent. The oxidized peptide solution was concentrated to a volume of about 10 ml and then chromatographed on Bio-Gel P4 in 1 M-acetic acid as solvent. The cyclic peptides were eluted slightly later on Bio-Gel P4 than their linear analogues which might be due to their more compact shapes. The cyclic peptides gave a single spot on t.l.c. in the solvent systems given under 'General procedures'. Their amino acid composition is given in Table 1.

β-Turn potentials of linear peptides

β-Turn potentials were calculated by using the predictive procedure of Chou & Fasman (1977). The relative probability of a tetrapeptide segment to form a β-turn is given as:

$$p_t = f_i \times f_{i+1} \times f_{i+2} \times f_{i+3}$$

where f describes the statistical frequency of an amino acid residue to be located in the corresponding position of the β-turn. Chou & Fasman (1977) have shown $p_t > 0.75 \times 10^{-4}$ to be a reasonable cut-off for a β-turn prediction. Helical and β-sheet probabilities were not considered.

General procedures

Dolichyl diphosphate [^{14}C]chitobiose was prepared on a large scale and purified as described earlier (Bause & Hettkamp, 1979). 5,5'-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) was used for the qualitative and quantitative determination of free thiol groups (Ellman, 1959). Radioactivity was detected either by radioautography on Sakura QH X-ray film or measured in a liquid-scintillation counter (Delta 300, Searle Analytic) using Bray's reagent as counting fluid (Bray, 1960). Amino acids were analysed on a Beckmann amino acid analyser, model 119 CL, after hydrolysis of peptides with 5.7 M-HCl in evacuated tubes for 24 h. T.l.c. was performed on silica gel with butan-1-ol/acetic acid/water (4:1:1, by vol.) and butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) as solvents.

Results

Characterization of acceptor peptides and time dependence of glycosyl transfer

Table 1 summarizes the amino acid composition of the various peptides which were used in this study. All peptides contain the 'marker sequence' Asn-Xaa-Thr and, with the exception of the hexapeptide Tyr-Asn-Gly-Thr-Ser-Val, two cysteine residues. The peptide chain length between the cysteine residues varies from two amino acids in peptide II up

to six in peptide I. The glycosyl acceptor properties were determined according to the standard assay procedure as described under Methods, using Dol-PP- ^{14}C chitobiose as glycosyl donor and a particulate membrane fraction of calf liver as enzyme source.

Because of the high susceptibility of the free thiol groups in the linear peptides towards oxidation, they were generally handled and stored in the more stable cyclic form. The glycosyl acceptor capabilities of the linear derivatives were measured after reduction of the corresponding cyclic peptides with an excess of dithiothreitol. Reoxidation was prevented by the addition of 5 mM-dithiothreitol to the incubation medium. Measurements with peptide VII as acceptor showed that the presence of dithiothreitol in the incubations had no effect on the activity of the *N*-glycosyltransferases.

A typical experiment characterizing the glycosylation of several cyclic and linear peptides as a function of time is represented in Fig. 1. The time course shows linearity of glycosyl transfer up to about 10 min. It was observed that in some cases substantial deviations from linearity occurred when longer incubation times were applied. This was shown to be mainly due to proteolytic activities present in the microsomal preparations. The hydrolytic breakdown of peptides could not be overcome by the addition of proteinase inhibitors like pepstatin, leupeptin or other inhibitors of serine proteinases. The results outlined in Fig. 1 indicate that the glycosylation rates are obviously dependent on the size of peptides and increase with increasing chain length. Strikingly, the cyclic derivatives seem to be poorer acceptors than their linear analogues. In the case of peptide VI, the cyclization is accompanied by a total loss of its glycosyl acceptor properties.

Comparison of glycosyl acceptor properties of cyclic and linear peptides in terms of K_m and V_{max}

In order to allow a better and more reliable interpretation of the differences in the glycosylation rates, we have determined K_m and V_{max} for the cyclic and linear peptides, respectively. The results of these measurements are summarized in Table 2. Included in the Table are also the relative glycosyl acceptor activities, given as V_{max}/K_m as well as the ratio of V_{max}/K_m for the linear and cyclic derivatives. The values for K_m and V_{max} represent average values from two separate determinations and are calculated from initial transfer rates by applying the procedure of Eadie and Hofstee (Eadie, 1942; Hofstee, 1952). The data were obtained with the same membrane preparation and a glycosyl donor of identical specific radioactivity and thus are directly comparable.

Table 1. Amino acid composition of synthetic acceptor peptides

Peptides (cyclic form) were hydrolysed with 5.7 M-HCl for 24 h in evacuated tubes. Values represent mean values of three separate determinations and are not corrected. For the amino acid sequences of the peptides see Table 2.

Amino acid	Residues in peptide:						
	I	II	III	IV	V	VI	VII
Aspartic acid	0.90 (1)	1.17 (1)	1.18 (1)	1.11 (1)	0.92 (1)	1.06 (1)	1.03 (1)
Threonine	1.07 (1)	1.12 (1)	1.12 (1)	1.21 (1)	0.98 (1)	0.97 (1)	0.98 (1)
Serine	1.08 (1)	—	1.07 (1)	1.06 (1)	0.95 (1)	—	0.97 (1)
Glycine	2.20 (2)	2.29 (2)	—	2.32 (2)	2.05 (2)	2.21 (2)	1.02 (1)
Cysteine	1.61 (2)	1.65 (2)	1.72 (2)	1.68 (2)	2.08 (2)	1.65 (2)	—
Valine	1.09 (1)	—	1.07 (1)	0.93 (1)	—	—	1.00 (1)
Tyrosine	0.93 (1)	0.79 (1)	0.75 (1)	—	—	—	0.94 (1)

Fig. 1. Time-dependency of glycosyl transfer from Dol-PP-[^{14}C]chitobiose to cyclic and linear acceptor peptides

Calf liver microsomes were incubated with 3300 c.p.m. of Dol-PP-[^{14}C]chitobiose in the presence of 1.15 mM of linear (filled symbols) or cyclic (open symbols) peptide, respectively. At the times indicated, the reactions were terminated by the addition of 1 ml of methanol and the [^{14}C]glycopeptides were isolated as described in the Materials and methods section. O, ●, Cys-Tyr-Asn-Gly-Thr-Ser-Val-Cys-Gly; □, ■, Cys-Asn-Gly-Thr-Ser-Val-Cys-Gly; △, ▴, Cys-Asn-Gly-Thr-Cys-Gly; ×, Tyr-Asn-Gly-Thr-Ser-Val.

The linear nonapeptide I was found to be the best glycosyl acceptor, as is documented by its highest V_{max}/K_m value. The shortening of this peptide on the C-terminal side of the 'marker sequence' by the

dipeptide unit Ser-Val (peptide II) results in an about 6-fold decrease in its relative glycosylation rate. This lower ability of peptide II to function as acceptor is mainly caused by an increase in K_m rather than a decrease in V_{max} . Omission of tyrosine on the N-terminal side (peptide IV) has a similarly detrimental influence. A further decrease of glycosyl-acceptor capabilities is caused by the additional shortening of the peptide chain, leading finally to an almost complete loss for peptide VI. (Peptide VII was included to permit a correlation with earlier studies.)

Cyclization of the linear peptides by disulphide bond formation is, with the exception of peptide III, generally accompanied by in part drastic increases in K_m as well as decreases in V_{max} . In fact, the initial transfer rates for the cyclic peptides I, II and V are impaired by about 15-fold in comparison with the linear derivatives. In contrast with that, the effect of cyclization is rather small on peptide IV, whereas for peptide III neither K_m nor V_{max} are affected. The latter peptide differs from the other cysteine-containing acceptors in that one cysteine residue is occupying the Xaa position of the 'marker sequence'. Disulphide formation leads, in this case, to the fixation of the asparagine residue only, whereas the C-terminal peptide segment including the threonine of the 'marker sequence' remains fully flexible. The observed influence of chain length on the glycosylation rates of the linear peptide acceptors appears to be mainly due to increases in K_m rather than decreases in V_{max} , whereas the opposite seems to be true for the cyclic derivatives.

Discussion

Recent studies in our laboratory have established that the hydroxy amino acid in the 'marker sequence' Asn-Xaa-Thr(Ser) plays a substantial role in the catalytic process of glycosylation (Bause & Legler, 1981). So we assume that its function is to

Table 2. *Glycosyl-acceptor properties of linear and cyclic peptides*

Glycosyl transfer was measured as described in the Materials and methods section. Apparent K_m and V_{max} values were evaluated by applying the procedure of Eadie (1942) and Hofstee (1952). They represent mean values of two separate experiments. n.d., not determined.

Peptide	Amino acid sequence	K_m (μ M)		V_{max} (c.p.m./min)		V_{max}/K_m		$[V_{max}/K_m]_{Lin.}$ $[V_{max}/K_m]_{Cyc.}$
		Linear	Cyclic	Linear	Cyclic	Linear	Cyclic	
I	Cys-Tyr-Asn-Gly-Thr-Ser-Val-Cys-Gly	50	340	480	250	9.6	0.7	13.5
II	Cys-Tyr-Asn-Gly-Thr-Cys-Gly	290	870	420	80	1.5	0.1	15.0
III	Cys-Tyr-Asn-Cys-Thr-Ser-Val	85	75	280	260	3.3	3.5	1.0
IV	Cys-Asn-Gly-Thr-Ser-Val-Cys-Gly	210	340	320	210	1.5	0.6	2.5
V	Cys-Asn-Gly-Thr-Ser-Cys-Gly	260	600	300	40	1.2	0.07	17.2
VI	Cys-Asn-Gly-Thr-Cys-Gly	n.d.	n.d.	n.d.	n.d.	0.2*	0.0*	—
VII	Tyr-Asn-Gly-Thr-Ser-Val	410	—	400	—	1.0	—	—

* Estimated from initial glycosylation rate.

enable the transfer of a proton from the side chain of asparagine onto a basic group at the active site of the *N*-glycosyltransferases. It is evident that any attempt to discuss conformational aspects with the various acceptor peptides has to take into account the extent to which the asparagine/threonine (serine) pair can fulfil this function.

The kinetic data, which are summarized in Table 2, allow several general statements. First of all, they show that the glycosylation rates of the linear peptides are strongly dependent on the peptide chain length. This finding points to an obviously essential character of the amino acids adjacent to the 'marker sequence' as is confirmed by earlier systematic studies on the same subject (Bause & Hettkamp, 1979). The elongation of the peptide chain, however, has a more pronounced effect on K_m rather than V_{max} , which suggests that the corresponding amino acids affect binding parameters rather than the catalytically important interaction between asparagine and the hydroxy amino acid within the 'marker sequence'. The influence of additional amino acids is less transparent for the cyclic derivatives. Here, the constraints imposed by disulphide formation apparently predominate over those effects which result from a corresponding alteration of the peptide chain length.

The cyclic peptides show, with the exception of peptide III, generally a lower ability to function as glycosyl acceptors than do their open-chain analogues. Obviously, cyclization results in a conformation that strongly impairs glycosylation. In fact, it seems that the restriction of the rotational degrees of freedom caused by disulphide formation renders a correct orientation between the particular asparagine and the threonine side chain more difficult or abolishes it altogether. The latter case applies to the

hexapeptide VI, which is the only peptide where glycosyl acceptor activity is not detectable. Extension of the ring by incorporating only one additional amino acid on either side of the 'marker sequence' (peptides II and V) which is associated with a corresponding increase in its flexibility, already results in peptide derivatives that are able to accept sugar, although the observed glycosylation rates are rather low (see the V_{max}/K_m values). Reasonable acceptor qualities require the further incorporation of amino acids into the peptide ring (peptides IV and I) without being able, however, to cancel the constraints resulting from disulphide bond formation.

Applying the predictive procedure of Chou & Fasman (1974) to amino acid sequences adjacent to carbohydrate-bearing asparagine residues of glycoproteins led to the observation that glycosylated asparagines had a high statistical probability of occurring in peptide regions that favour the formation of β -turn or other loop structures (Aubert *et al.*, 1976; Beeley, 1978). Proceeding on this assumption we have tried, by the use of space-filling models, to develop conformational concepts that fit the above statistical as well as the various experimental results described here and in earlier studies.

The simulation of a β -turn conformation with space-filling models reveals that the particular hydrogen bond interaction between the side chains of asparagine and the hydroxy amino acid is only capable of being produced when the asparagine residue occupies the first (*i*) or third (*i* + 2) position of the β -turn, whereas a contact between both amino acids is obviously not possible with asparagine being located in the second (*i* + 1) position. This observation indeed agrees fairly well with the statistical frequencies of occurrence of glycosylated aspara-

gine residues in the corresponding positions of a β -turn, as reported by Beeley (1977). It seems, however, that the contact between asparagine when it is located in the $(i+2)$ position of the β -turn and the hydroxy amino acid is critically dependent on the mutual orientation of the peptide segments which are joined with the β -turn. Thus, it follows from the model that a hydrogen bond interaction within the 'marker sequence' cannot be arranged when the peptide chain folds back on itself by 180° by forming regular antiparallel sheet structures that are stabilized by the maximum number of hydrogen bonds. In this case, the side chain of the hydroxy amino acid is directed 'inwards' with the consequence that a contact between the amino acids of the 'marker sequence' is prevented. This restriction concerning the mutual orientation of asparagine and the hydroxy amino acid, imposed by the specific structure-forming potentials of the peptide segments framing the β -turn, is inoperative when the folding back of the peptide chain occurs by a loop that involves five instead of four amino acids ($i+4$ loop). Concerning the location of asparagine in the (i) and $(i+1)$ position, however, the above considerations discussed for the β -turn are fully transferable to an $(i+4)$ loop structure. In fact, this study indicates that the formation of a carbohydrate attachment site is not only dependent on the presence of the 'marker sequence' within a β -turn or loop, but obviously requires a complex interplay among various segments of the peptide chain according to their structure-forming potentials.

The space-filling model of the linear hexapeptide Cys-Asn-Gly-Thr-Cys-Gly, which is represented in Fig. 2 as a photograph, gives a rough idea of how the hydrogen bond interaction between the side chain of asparagine and the hydroxy amino acid might look like. The peptide chain is here arranged as an $(i+4)$ loop structure with asparagine in the first (i) position. It is stabilized by a hydrogen bond between the α -carbonyl of asparagine (i) and the α -amide of the C-terminal glycine $(i+4)$. The distance between the two cysteine residues in this presumably 'active' conformation is rather large, which suggests that the cyclization of the peptide via disulphide bond formation will not be possible without breaking the contact between asparagine and threonine. In fact, this can be deduced from the peptide model and is confirmed experimentally by the lack of acceptor properties of the cyclic derivative (see Table 2). In agreement with the experimental results, the model of the heptapeptide Cys-Tyr-Asn-Gly-Thr-Cys-Gly (Fig. 3) demonstrates that, obviously due to the higher flexibility, a correct orientation within the 'marker sequence' is not prevented by disulphide formation. However, the rather low transfer rate for this peptide indicates that this conformation might be less favoured.

Peptide III, the only peptide that exerts identical glycosylation rates in its linear and cyclic form, differs from the other cysteine-containing peptides by the fact that not the complete 'marker sequence', but asparagine only is framed by the cysteine residues. In this configuration, the C-terminal peptide

Fig. 2 Space-filling model of the linear hexapeptide *Cys-Asn-Gly-Thr-Cys-Gly*

The peptide chain is arranged in a turn structure being stabilized by hydrogen bonding between the α -carbonyl of asparagine (i) and the α -amide of the C-terminal glycine $(i+4)$. The thiol groups and the hydroxyl function of threonine and the β -amide of asparagine, which are connected by a hydrogen bond, are indicated by arrows.

Fig. 3. Space-filling model of the cyclic heptapeptide *Cys-Tyr-Asn-Gly-Thr-Cys-Gly*. Structural features are indicated as in Fig. 2.

Fig. 4. Model of the cyclic heptapeptide *Cys-Tyr-Asn-Cys-Thr-Ser-Val*. Structural features are indicated as in Fig. 2.

'tail', including the catalytically active threonine, remains flexible. This flexibility seems to be great enough to surpass any negative effects resulting from the fixation of asparagine after cyclization. The simulation of this peptide in a model (Fig. 4) indeed illustrates that the intramolecular oxidation, as well as the reduction, of the cysteine residues can occur without any interference with, or rearranging of, the peptide backbone, as is required for peptide VI.

We have calculated, by applying the predictive procedure of Chou & Fasman (1977), the β -turn-forming potentials of linear acceptor peptides for all tetrapeptide segments which contained the asparagine residue of the 'marker sequence'. The predictive Chou and Fasman method is based on observed frequencies of the occurrence of various amino acids in α -helical, β -sheet and β -turn regions of proteins, the structures of which are known from

X-ray crystallographic data. Our calculations, the results of which are not shown in detail, revealed a generally high statistical potential for the particular asparagine to be involved in the formation of β -turn or loop structures. At first sight, this elevated structure-forming potential appears to be rather meaningless, because shorter peptides exist in aqueous solution as a statistical mixture of flexible conformers. It is well known, on the other hand, that even small peptides may assume an ordered conformation when they are transferred into a hydrophobic environment or into a solvent less polar than water. As the binding process of the acceptor peptides to the active site of *N*-glycosyltransferases is accompanied by an alteration of their hydrophilic environment, it is likely that the 'active' conformation is being induced as the binding process is progressing. In that case it is imaginable that the adoption of an ordered 'active' conformation might be facilitated due to the corresponding structure-forming potentials of peptides. The idea of an 'induced conformation' would, at least for studies *in vitro* with shorter acceptor peptides, delineate a plausible explanation that alterations in the peptide region adjacent to the 'marker sequence' obviously affect binding parameters rather than $V_{\max}$. Disulphide bonds, on the other hand, would hinder or prevent the full development, of this specific conformation, which might explain the similar, but substantial, decrease in the relative glycosylation rates of some cyclic peptides in comparison with their linear analogues.

The various results taken together show that the formation of a carbohydrate attachment site is regulated and presumably determined by a combination of several factors. Fundamentally, interaction between asparagine and the hydroxy amino acid of the 'marker sequence' must be possible. This is realized optimally when the peptide chain can adopt a conformation resembling a β -turn or other loop structures. The induction of the 'active' conformation requires additional amino acids on both the *N*- and *C*-terminal side of the 'marker sequence'.

We thank Dr. K. von Figura (Münster) for samples of pepstatin and leupeptin. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

- Aubert, J. P., Biserte, G. & Loucheux-Lefebvre, M. H. (1976) *Arch. Biochem. Biophys.* **175**, 410–418
- Bause, E. (1979) *FEBS Lett.* **96**, 179–182
- Bause, E. & Hettkamp, H. (1979) *FEBS Lett.* **108**, 341–344
- Bause, E. & Legler, G. (1981) *Biochem. J.* **195**, 639–644
- Bause, E. & Lehle, L. (1979) *Eur. J. Biochem.* **101**, 531–540
- Beeley, J. G. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1051–1055
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Chou, P. Y. & Fasman, G. D. (1974) *Biochemistry* **13**, 222–245
- Chou, P. Y. & Fasman, G. D. (1977) *Adv. Enzymol.* **47**, 45–148
- Eadie, G. S. (1942) *J. Biol. Chem.* **146**, 85–93
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **92**, 70–77
- Erickson, B. W. & Merrifield, R. B. (1976) in *The Proteins* (Neurath, H. & Hill, R. L., eds.), vol. 2, pp. 255–562, Academic Press, London and New York
- Hofstee, B. H. J. (1952) *J. Biol. Chem.* **199**, 357–364
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Marshall, R. D. (1967) *Abstr. Int. Congr. Biochem.* 7th, pp. 573–574
- Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154
- Moulé, Y., Roullier, C. & Chaveau, J. (1960) *J. Biophys. Biochem. Cytol.* **7**, 547–558
- Neuberger, A. & Marshall, R. D. (1968) in *Carbohydrates and their Roles* (Schultze, H. W., Cain, R. F. & Wrothstad, R. W., eds.), p. 115, Avi, Westport, CN
- Pless, D. D. & Lennarz, W. J. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 134–138
- Ronin, C., Granier, C., Van Rietschoten, J. & Bouchiloux, S. (1978) *Biochem. Biophys. Res. Commun.* **81**, 772–778
- Struck, D. K. & Lennarz, W. J. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., ed.), pp. 35–84, Plenum Press, New York and London