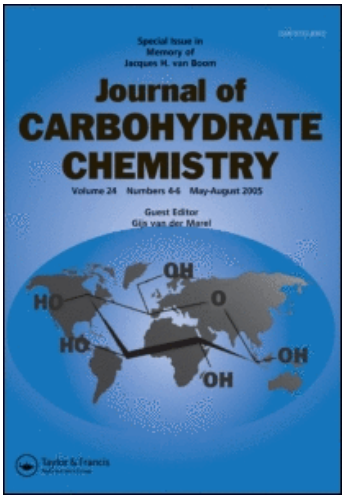

Journal of Carbohydrate Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617200

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To cite this Article Feeney, Robert E. , Osuga, David T. and Yeh, Yin(1994) 'Chemical and Enzymatic Derivatization of Carbohydrate Side Chains of Antifreeze Glycoproteins', Journal of Carbohydrate Chemistry, 13: 3, 347 — 361
To link to this Article: DOI: 10.1080/07328309408009198
URL: http://dx.doi.org/10.1080/07328309408009198

J. CARBOHYDRATE CHEMISTRY, 13(3), 347-361 (1994)

REVIEW ARTICLE

CHEMICAL AND ENZYMATIC DERIVATIZATION OF CARBOHYDRATE SIDE CHAINS OF ANTIFREEZE GLYCOPROTEINS

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Received July 13, 1993 - Final Form January 4, 1994

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1. Introduction

1.1. Types of Antifreeze Proteins

At this time there have been a variety of different fully or partially characterized proteins that have the function described as "antifreeze."[1-10] The proteins that have been studied for the longest time are antifreeze glycoproteins (AFGP). These are polymers of H_2N[Ala-Ala(β-galactosyl(1->3)α-*N*-acetylgalactosamine)Thr]$_n$Ala-Ala-COOH (Figure 1), and they typically consist of a number of distinct components. Eight have been characterized (n = 50, 45, 35, 28, 17, 8, 5, and 4) AFGP 1 to AFGP 8, respectively. The small antifreeze polymers, AFGP 6-8, constitute over 80% of the total proteins and have some prolines following threonines.[11] These are found in the antarctic fish families of *Nototheniidae, Channichthyidae,* and *Bathydraconidae,* as well as in the arctic fish, *Boreogadus saida, Gadus morhua, Gadus ogac,* and *Microgadus tomcod.* The arctic fish *Eleginus gracilis* also has very similar glycoproteins (EgAF) except that the major fractions,

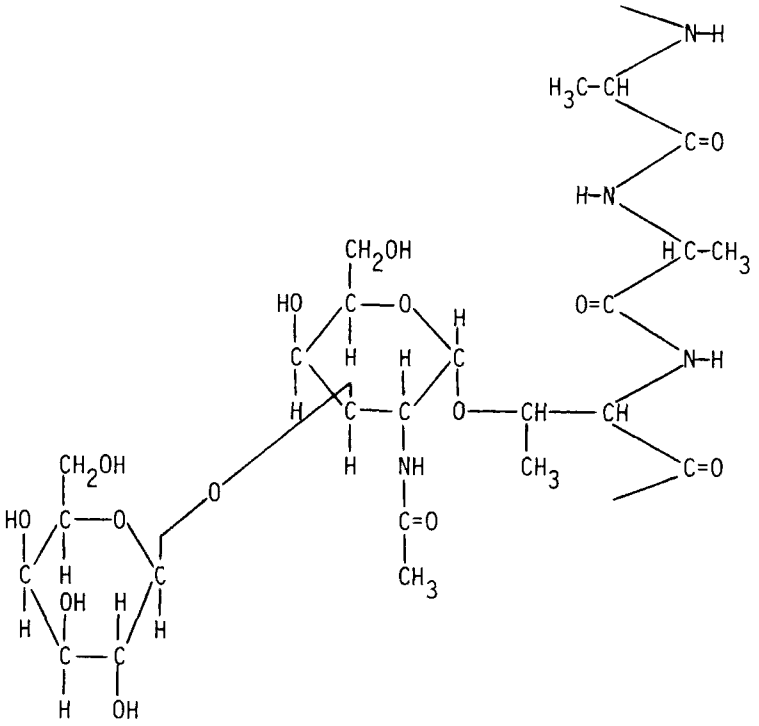

Figure 1. Polymer unit of antifreeze glycoproteins 1-5 (from Feeney.[8]).

the smaller ones (EgAF 7R and EgAF 8R), have additional dipeptides at the C-terminal end consisting of arginine and alanine[12] (Figure 2).

In addition to antifreeze activity, the longer AFGP fractions (1-5) inhibit hemagglutination by osage orange lectins.[13] The short fraction, AFGP 8, however, had only a very weak activity (< 2% of that of AFGP 1-5). In this paper, this activity is termed 'antilectin activity'.

There are other antifreeze proteins that contain no carbohydrate.[7,10] Some of these, like the winter flounder (*Pseudopleuronectes americanus)* antifreeze protein (AFP), are very similar to the glycoproteins in amino acid content. Nearly two-thirds of their amino acids are alanine, but they have other amino acids, mostly hydrophilic, instead of the carbohydrate. AFP has now been synthesized[15] and its crystal structure at 2.5 Å resolution has been reported.[16] There are still others that are more normal-like proteins, but their characterization is still in progress.[7-10]

Pagothenia borchgrevinki 8

NH_2-Ala-Ala-Thr-Ala-Ala-Thr-Pro[a]-Ala-Thr-Ala[b]-Ala-Thr-Pro-Ala-COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Boreogadus saida 8

NH_2-Ala-Ala-Thr-Pro-Ala-Thr-Ala-Ala-Thr-Pro-Ala-Thr-Ala-Ala-COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Eleginus gracilis 8R

NH_2-Ala-Ala-Thr-Ala-Ala-Thr-Pro-Ala-Thr-Ala-Ala-Thr-Pro-Ala-Arg-Ala-COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Pagothenia borchgrevinki 7

NH_2-Ala-Ala-Thr-Ala-Ala-Thr-Ala[c]-Ala-Thr-Ala[d]-Ala-Thr-Ala[c]-Ala-Thr-Pro-Ala-COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Boreogadus saida 7

NH_2-Ala-Ala-Thr-Pro-Ala-Thr-Ala-Ala-Thr-Pro-Ala-Thr-Ala-Ala-Thr-Pro-Ala-COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Eleginus gracilus 7R

NH_2-Ala-Ala-Thr-Ala-Ala-Thr-Pro[e]-Ala-Thr-Ala-Ala-Thr-Pro-Ala-Thr-Ala-Ala-Arg-Ala-COOH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Residue interchange with:

a. Ala in ratio Pro:Ala = 7:3
b. Pro in ratio Ala:Pro = 8:2
c. Ala in ratio Ala:Pro = 2:1
d. Pro in ratio Ala:Pro = 9:1
e. Ala in ratio Pro:Ala = 1.25:1

Figure 2. The complete primary sequences of the smaller (shorter) antifreeze glycopeptides from the fish *P. borchgrevinki*, *B. saida* and *E. gracilis* (Feeney et al.[8]).

1.2 Conformation of antifreeze glycoproteins

Physically, AFGP molecules exhibit spectrosiopic data consistent with the structure of a three-fold, left-handed helix, but long-range order may be low because of segmental mobility.[17-20] Theoretical analysis indicates that this type of helix is energetically stable.[21] Dill et al.[22] have recently reported the presence of NOE cross-peaks between GalNAc and the peptide backbone suggesting the proximity of these groups.

In our model [19] the spectroscopically derived data were combined with conformational energy calculations to give a conformational model for antifreeze glycoprotein in which the

hydrophobic surfaces of the disaccharide side chains are wrapped closely against a three-fold left-handed helical peptide backbone. The hydrophilic sides of the disaccharides were aligned so that they may bind to the ice crystal facet that is perpendicular to the fast growth axis, inhibiting normal crystal growth. The three-fold single helix was favored by Sansom et al.,[23] who used computer modeling based on a linked-atom-least-squares system to examine three stereochemically feasible polypeptide conformations: the alpha helix, the beta chain, and the three-fold left-handed single helix. In contrast, a γ-turn structure has recently been suggested by Drewes and Rowlen [24] based on vibrational spectroscopy and computer-generated molecular models In another study by Atkins and Sansome [25] using Monte Carlo modeling of the interaction of AFGP with water, a mechanism for antifreeze action was proposed based on the formation of a non-ice-like structure by water molecules immediately surrounding the AFGP molecule. As Feeney and Yeh discussed in 1978[1], there is a periodicity in the ice Ih structure where twice the a-axis repeat distance is approximately the same as the periodicity of the AFGP molecule (0.936 nm). Wen and Laursen,[26] however, have shown that the fitting of antifreeze proteins on the ice surface is complex by finding that an enantiomer (synthesized from d-amino acids) of a non-glycoprotein antifreeze protein can also inhibit ice crystal growth.

Other studies have been with chemically synthesized glycopeptides related to antifreeze glycoproteins. In one, the gycopeptides were reported to form an intramolecular hydrogen bond between the amide-proton of *N*-acetylgalactosamine and the carbonyl oxygen of threonine to which the *N*-acetylgalactosamine was covalently linked.[27] In another study similar circular dichroism spectra were found for natural antifreeze glycoproteins and the synthetic glycopeptides [Thr(B-D-galarose)-Ala-Ala]$_n$.[28]

1.3 Inhibition occurs at ice surface

AFGP lowers the freezing temperature ~500 times more than predicted from solution colligative properties, functions additively with salt and in the presence of ice. All the physical and chemical examinations show no unusual reactions between AFGP molecules and water,[1-5] and there is no evidence that these molecules affect the structure of ice once it is formed. The remaining possibility is that it functions at the ice-solution interface and, indeed, all data appear to support such an action.[5]

Functional studies of the AFGP-water system[29] have shown that all AFGP components examined have a plateau in activity at high concentration, but the actual value of the plateau activity differs between the different length AFGP components. While the low molecular weight component (AFGP 8) loses activity at deep supercooling, at very high concentrations activity is restored. The activity data fit a reversible kinetic model of AFGP activity, and the coefficients obtained can be used to compare the activity differences

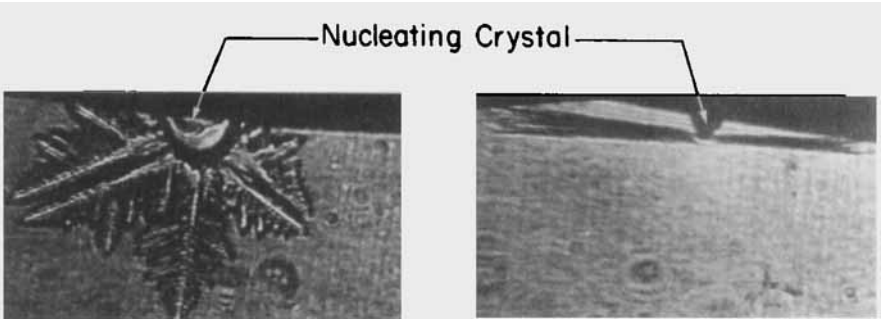

Figure 3. Crystal growth of ice in pure water (left) and in 5 mg/ml antifreeze glycoprotein (AFGP) solution (right) at -1.25°C. Growth was from a single oriented crystal of ice inserted in the solution. On the left the c axis is normal to the plane of the page; growth is along the a axis. On the right the a axis is normal to the plane of the page; growth is along the c axis (from Harrison et al.[34]).

between the AFGP components as well as between AFGP and EgAF. Furthermore, with proper equilibration there are only minute differences (decreases) of activity in D_2O as compared to H_2O.[30]

Although many observations indicate that AFGP functions at the ice-solution interface, probably the most important data to date are those providing direct evidence for AFGP adsorbing to an ice surface: In one experiment, the AFGP-ice system exhibit inversion asymmetry across the ice solution interface and this was detected by surface second harmonic generation, presenting almost absolute evidence for the adsorption of AFGP on, or at, the surface of the ice.[31] Other data have shown that the interfacial energy is lowered at the ice-solution interface.[32] Several other studies have shown that crystals growing into an AFGP solution have an unusual form.[33-35] A striking difference was noted when oriented seed crystals were introduced into supercooled solutions of AFGP. In solutions of AFGP with a seed crystal oriented with its c axis perpendicular to this direction (in the plane of the page), crystals grew unequivocally parallel to the c axis.[34,35] On the other hand, in pure water a seed crystal with the c axis out of the page gives rise to a crystal growing as a thin dendrite in the plane of the photograph (Figure 3). These results contrasted with the control protein studies, where ovomucoid in solution lead to ice growth the same as that found in pure water. The linear growth rate of ice in AFGP solutions can be as much as five times greater than in water, depending upon the extent of supercooling when seeding occurred.

In early tests of activity of AFGP, solutions were supercooled and nucleated by a vibrating probe. When the shorter glycoproteins AFGP 7 or 8 are tested by this nucleating

method at temperatures <-3 °C, little or no activity is observed whereas at the same concentrations, measurable activity is observed on nucleation at temperatures > -1.0 °C.[36] However, when a small amount of the longer AFGP is added together with the AFGP 8, an equivalent of the activity of AFGP 8 is expressed on nucleation at temperatures < -3.0 °C. Thus far, no evidence for interaction between the two species has been observed in solution. This cooperativity was also not found between AFGP 8 and the non-glycoprotein, AFP.

Other studies have shown that the loss of activity of the low molecular weight AFGP 8, and its retention in the presence of the longer polymer on nucleation at temperatures < -3 °C, may be due to the growth habits of the ice crystal resulting from the degree of supercooling[37] and not necessarily due to the rate of ice crystal growth during the actual measurement of freezing temperature.[38] In other words, it is the number of growth sites that needs to be covered that defines functional activity.

1.4 Inhibition is by a kinetic adsorption-desorption process

From these and other results, Harrison et al.[34] have suggested that at temperatures warmer than the freezing temperature, AFGP molecules block the growth of ice crystal perpendicular to the c axis, as well as inhibiting surface nucleation necessary for c-axis growth on the basal plane. Below the freezing temperature nucleation and growth occur along the c-axis direction, bipyramids and thin needles growth have been seen under nucleation and growth conditions respectively. The needle tip growth is favored because of a combination of good thermal dissipation along the ice crystal while its lateral growth is still blocked by adsorbed molecules. With further temperature lowering, growth along the a axis eventually reverts to its dominant role and "normal" growth behavior continues. AFGP molecules block growth only as long as they have time to adsorb and block water molecules' adsorption onto the same site, this being the result of a competitive process. Overall, one could describe this as a kinetic interaction at the growth-inhibited surface, either blocking the adsorption of water molecules or scavaging incipient growth structures.[8]

All these results, therefore, confirm our original suggestion made in 1972 that the mechanism of action "probably involves an interface separating solid and liquid during growth of ice crystals".[39] Yang *et al.*,[16] reporting on the x-ray crystallographic structure of the nonglycoprotein antifreeze (AFP) from the winter flounder, have suggested that this protein would hydrogen bond to the ice surface. In continuing the various previous studies on the effects of antifreeze proteins on the habits of ice crystal growth,[32-35] Raymond et al.,[40] reported that, as new layers are deposited on the basal plane, pyramidal surfaces develop on the outside of the crystal and large hexagonal pits form within the basal plane. The authors interpreted these effects as being due to growth inhibition on the prism, pyramidal and pit faces derived from adsorption of the antifreeze.

Table 1. Carbohydrate Modifications of Antifreeze Glycoproteins 1-5[a]

Treatment			
Reagent	Reaction	Modification (%)	Antifreeze Activity Remaining (%)
Alkaline I_2 oxidation	None	0	>90
Acetic anhydride in pyridine	Acetylation of carbinols (and amino terminal	32	18
Acetylation , then hydroxylamine	Deacetylation	>90	>90
Alkaline periodate	Oxidative scission of galactose	90	<15
High pH	β-Elimination of disaccharide and racemization	80	<10
Enzyme + sialic acid	Sialylation of carbinols	30(?)	0
Sialytion, then Neuraminidase	Desialyation of carbinols	>80	80
Galactose oxidase	Oxidation of C-6 carbinols	~80	>80
Galactose oxidase, then excess bisulfite	Formation of aldehyde - bisulfite complex	Excess bisulfite	17
Galactose oxidase, then alkaline I_2 oxidation	Oxidation of C-6 carbinols to carboxyls	70	15
Galactose oxidase, then glycylglycine and cyanoborohydride	Amination of aldehydes with amino terminal of glyclglycine	50	88

a. Adapted from Feeney and Osuga.[43]

2. Derivatizations Of Carbohydrate

2.1 Purpose of modifications

Chemical modification of proteins is done for many different purposes.[41] Of these, long-used modifications[42] are those to change properties in order to determine the role of structure in function, i.e., structure-function relationships. For determining the role of an individual amino acid residue, *in vitro* mutagenesis is now the frequent method of choice. With carbohydrate side chains the *in vitro* mutagenic approach is not easily applicable. Modifying proteins, in general, with chemical reagents may present difficulties in detection of nonspecificities and in quantitations. With carbohydrate side chains even more difficulties are present because of the limited number of reactions suitable for the carbohydrate moieties in glycoproteins.

The objective in modifying the carbohydrates of AFGP has been to determine how changing the carbohydrate would affect function. To this end, several modifications have been done to remove all or part of the carbohydrate, to add a charge to it, to add hydrophobic groups, and to add amino acids and peptides at the C-6 position of the galactosyl group (Table 1).

2.2 Inhibition by boronic acid ions

Sodium borate strongly inhibited antifreeze activity at a pH where the salt was ionized (pH 9). In recent studies with organic boronic acids, low binding but strong inhibition,

Table 2. Effect of Time on β-Elimination of Antifreeze Glycoprotein on Losses of Threonine and Galactosamine[a]

Time, h	Threonine loss, %	Galactosamine loss, %
0	0	0
0.25	16	22
1	24	30
2	46	53
4	59	58
7	61	68
10	65	73
24	65	73
48	86	92

a. Reaction was done at 6 mg of AFGP-8/mL of 0.5 N NaOH at 37 °C. Norleucine was added as internal standard in the amino acid analyses. From Lee et al. (45) with permission.

was found.[30] In contrast, the antifreeze activity of a non-glycoprotein antifreeze from *Pseudopleuronectus americanus* was not inhibited and the antilectin activity of the AFGP was unaffected.

2.3 Acetylation with acetic anhydride

Acetylation of hydroxyl (and the one terminal amino group) with acetic anhydride in pyridine buffer, pH 9.0, caused extensive losses in activity. Subsequent treatment with hydroxylamine resulted in complete removal of the acetyl group from the hydroxyls and > 95% recovery of activity.[44] Antilectin activities were affected similarly.[13]

2.4 Alkaline periodate oxidation

Oxidation of AFGP components with 0.01 M periodate at 4 °C for 5 hours caused complete loss of antifreeze activity.[44] Upon reduction of the oxidized products with sodium borohydride and cleavage of the acetal bonds with 0.05 M H_2SO_4 at 80 °C for 1 hour, only 18% (three residues) of the galactose remained intact. No loss of galactosamine, threonine or alanine was detected.

Most of the antifreeze activity (84%) and the antilectin activity (89%) was destroyed when 90% of the galactose was destroyed by a similar periodate oxidation.[13]

2.5 Treatment at high pH (β-Elimination)

β-Elimination of the disaccharide destroyed both the antifreeze and antilectin activities.[13] When 80% of the carbohydrate was β-eliminated, approximately 10% and 20% of the antifreeze and antilectin activities, respectively, remained. Losses in threonine and galactosamine correlated well (Table 2).[45]

β-Eliminations of AFGP-8 were also studied as a model system for β-eliminations of O-threonine substitutes.[45] These include the effects of time, temperature and pH on the β-elimination and additions of sulfite and N^{α}-acetyl-L-lysine to the β-eliminated product. An activation of energy of 9.60 kcal/mol substituted threonine was calculated, a value about half of those obtained for most disulfides and O-phosphoseryl residues. At pH 10, there was about a 75% loss of threonine and a 70% addition of β-methyldehydroalanyl residues with sulfite. With N^{α}-acetyl-L-lysine, after acid hydrolysis two chromatographic peaks suspected as the D and L isomers of N^{ϵ} - (1-methyl-2-amino-2-carboxyethyl)-L-lysine were found.

2.6 Attachment of Sialic Acid

Sialic acid (NAM) was enzymatically attached to AFGP 2-3 by use of a sialytransferase to give 95% modification of the galactoses. The product (NAM-AFGP) was < 5% active.[46] On desialylation of the NAM-AFGP with the enzyme neuraminadase the activity was regained.[46] Comparative studies with different sialotransferases have recently been done with AFGP and several other glycoproteins.[47]

2.7 Enzymatic oxidation of C-6 hydroxyls to aldehydes with galactose oxidase

Oxidation of the C-6 hydroxyls of the galactoses with galactose oxidase did not destroy activity.[13,39,48,49] When the C-6 hydroxyls were oxidized to aldehydes with galactose oxidase in a preparative experiment, 79% of the terminal D-galactose and 19% of the D-*N*-acetylgalactosamine were oxidized with > 80% of both antifreeze and antilectin activities still remaining.[13,39]

Five different preparations of the aldehydo product also differed from the original in a physical property of solubility. Although they were as soluble as the original, they dissolved at much slower rates.

2.8 Oxidation of aldehydes of enzymatically oxidized AFGP to carboxyl groups with alkaline I_2

Oxidation of the aldehydes (produced enzymatically) to the carboxyls caused extensive loss of activity, giving a product with only 20% activity.[10,34] Antilectin activity was affected similarly.[13] But when assays were done in acidic solutions (pH 2) to protonate the carboxyls, an antifreeze activity of 45% was observed.

2.9 Formation of Bisulfite Adduct of Oxidized Product

The addition of bisulfite to the galactose oxidase oxidized AFGP caused extensive decreases in both antifreeze activity and antilectin activity.[13,39] The inactivation was reversed on the removal of the sulfite.

Table 3. Modification of Carbohydrate Moieties of Antifreeze Glycoproteins: Substitution at C-6 Positions with Amino Acids and Peptides[a,b]

	Amino acids found per[c]			Residues	
Modification		Disaccharide	Mole	per mole	Activity (%)[d]
Gly	Gly	0.15	2.9	?	64
$(Gly)_2$	Gly	0.40	8.0	5.0[e]	88
$(Gly)_3$	Gly	0.67	13.4	5.3[e]	82
$(Gly)_4$	Gly	0.87	17.4	4.8[e]	70
$(Gly)_2$-NH_2	Gly	0.08	1.7	<1.7	44
Gly-Phe	Gly	0.11	2.2	5.8	60
	Phe	0.29	5.8		
$(Gly)_2$-Phe	Gly	0.36	7.2	5.2	30
	Phe	0.26	5.2		
Gly-Tyr[f]	Gly	0.26	5.2	9.9	57
	Tyr	0.50	9.9		
Gly-Leu	Gly	0.11	2.1	5.4	40
	Leu	0.27	5.4		
$(Gly)_2$-Leu	Gly	0.44	8.8	5.8	50
	Leu	0.29	5.8		
$(Gly)_2$-Val	Gly	0.34	6.8	5.9	35
	Val	0.30	5.9		
Gly-Glu	Gly	0.09	1.8	5.2	13
	Glu	0.26	5.2		
Arg[g]	Arg	0.42	8.4	8.4	30

a. From Osuga et al. (1989)[49] with permission.
b. C-6 position of galactosyl (and some *N*-acetylgalactosyl) groups first oxidized with galactose oxidase. The resulting carbonyl groups were then aminated with amino acids or peptides by reduction with cyanoborohydride.
c. Amino acid analyses were done by amino acid analysis after acid hydrolysis.
d. Calculated on activity of oxidized preparations.
e. Estimated values for substitution by Gly peptides per glycotripeptide when value (0.15) for single Gly subtracted.
f. Value for tyrosine also done by spectrophotometry on intact protein: 0.49.
g. Value for arginine was also determined colorimetrically on intact protein with *p*-nitrophenylglyoxal.

2.10 Attachment of amino acids and peptides to C-6 hydroxyls by reductive amination of galactose oxidized AFGP

Amino acids and peptides were attached to the C-6 hydroxyls of the galactose and the *N*-acetylgalactosamine by first oxidizing the C-6 hydroxyls to the aldehydes by galactose oxidase in the presence of small amounts of catalase followed by reductive amination (α-amino group of amino acid) in the presence of cyanoborohydride.[49] The activity of oxidized antifreeze glycoprotein was > 70% of the original, and considerable activity was retained with some substitutions on reductive amination using cyanoborohydride. The activities retained (as compared to the oxidized antifreeze glycoprotein) are listed in Table 3. Similar activities were also obtained at the very low

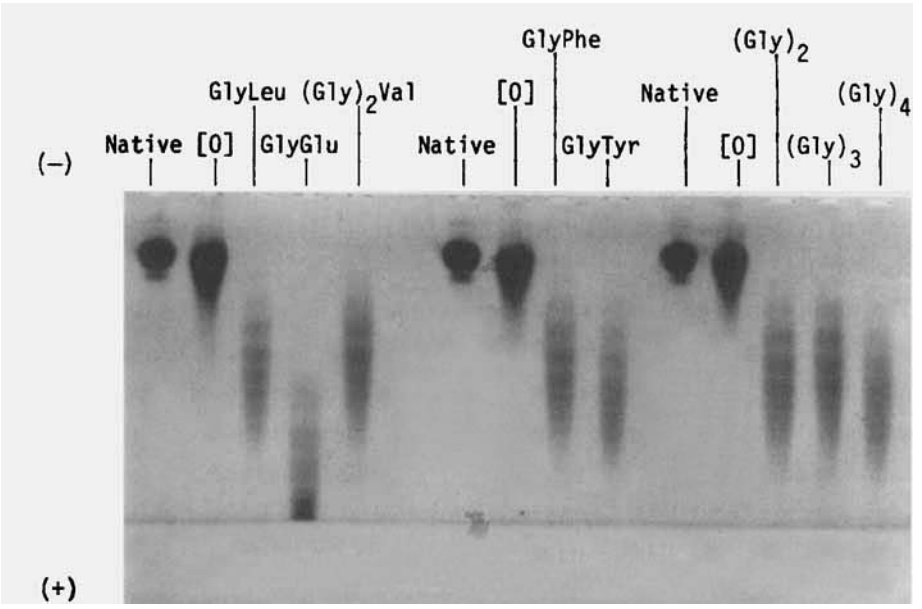

Figure 4. Acrylamide gel electrophoretic pattern of native, oxidized [O], and peptide-derivatized oxidized antifreeze glycoproteins (from Osuga et al.[49]).

concentrations required for inhibition of recrystallization.[50] On amino acid analysis of acid hydrolysates, some release of the amino acid attached by amination occurred; e.g., Gly-Tyr gave 0.26 Gly and 0.49 Tyr per disaccharide. The product of Gly-Tyr (with a charge change) was examined electrophoretically along with the native and the oxidized intermediate (Figure. 4). A slight fuzziness in the oxidized intermediate but no native or intermediate material was observed in the aminated derivative.

3. Conclusions On Role Of Carbohydrate

Chemically modified proteins are rarely completely homogeneous due to nonspecific interactions, the presence of some of the original unmodified material, or incomplete modifications of all the available possible reactive groups. With AFGP the large numbers of hydroxyl groups increase the possibilities of incomplete modifications. Separations of these types are rarely accomplished. Most of the analyses give figures for total groups modified and so do not differentiate between these types. For example, a 40% modified product could contain some inactive material that was > 60% modified, and some active

material that was < 10% modified; an assay value of 20% activity could be due to only the material < 10% modified.

The acetylations show that placing the more hydrophobic and larger acetyl group on the hydroxyls causes loss of activity. The β-elimination results are inconclusive because some racemizations occur, but they indicate the importance of the carbohydrate side chains. The scissions of the carbohydrates by periodate oxidations also indicate the importance of the carbohydrates.

The results that appear the most interpretable are those with the oxidations by galactose oxidase and the subsequent derivatizations with amino acids. Oxidations of the C-6 to the aldehyde definitely caused minor losses, and attachments of amino acids and peptides caused surprisingly only small losses with most substitutions. (There is no significant net charge change adding a secondary amino group and a carboxyl group, mainly just a steric effect.) With a tetraglycyl derivative it would appear that the C-6 group of the galactosyl residue does not interact directly on the ice surface. It is attractive to postulate that this data might be extrapolated to conclude that the AFGP positions itself with its hydrophobic side on the ice and the sugars in the solution.

Notwithstanding the results with the addition of peptides to the C-6 position, the general interpretation is that the carbohydrate side chains interact with the ice surface.[1-10] Recent experiments showing how the short AFGP 8 might interact with ice also supports a role of the carbohydrate in essential agreement with calculation of Feeney and Yeh[1] showing a relationship between the periodicities of the AFGP molecule and the a-axis repeat distance of ice.[51] Jorgensen, *et al*,[52] in a simulation of the structure of the non-glycoprotein AFP, reported values of spacing between the oxygen atoms of hydrophylic hydroxyl groups of the threonines close to the repeat distance of 16.6 Å between oxygen atoms along the [0012] direction in ice. In general, the chemical derivatizations show that the carbohydrate is important for the antifreeze functions, but the derivatizations do not show that the carbohydrate in AFGP is itself involved in the inhibition of the ice crystal growth. Rather, the derivatizations indicate a necessity of the hydrophilic character of the molecule. Further studies are needed to more accurately characterize the role of the carbohydrate, either as involved in the interaction with the ice surface or as a hydrophilic assistant. Direct physical observation of how AFGP is positioned on the ice is necessary.

The mechanisms of action of both the antifreeze glycoprotein and its opposite, the bacterial ice-nucleating protein, could involve interactions with clusters of water molecules or at the solution interface of such clusters (or incipient ice crystals).[8] In the case of the antifreeze glycoproteins, interaction might occur with some clusters (or incipient ice crystals), leading to their dissociations to water. In the case of the ice-nucleating proteins, they might cause further development of the clusters to ice and the ensuing crystallization.

Acknowledgements

This work was supported in part by National Institutes of Health Grant GM 23817. The authors would like to thank Barbara Smith, Chris Howland, Diana Melbourn, Clara Robison and Eric Hamilton for editorial assistance and typing of the manuscript.

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