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Comparative study of the asparagine-linked oligosaccharide structures of normal and acute-phase rat plasma thiostatin

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Rat plasma thiostatin is a 68 kDa glycoprotein with kinin donor and cysteine proteinase inhibitor properties. Thiostatin is an acute-phase plasma protein (APPP) with dramatically elevated plasma levels in response to inflammatory stimuli. APPPs have been shown to possess altered glycan structures in inflammation. This study compares the carbohydrate structure of normal thiostatin with that expressed during the acute-phase response. Thiostatin from both normal and acute-phase plasma was purified by carboxymethyl-papain Sepharose 4B affinity chromatography. Sugar composition analysis by gas chromatography and the Warren method yielded similar mean values for both proteins on a mole sugar per mole protein basis (normal/acute phase): fucose, 2.4/1.7; mannose, 7.5/8.0; galactose, 11.2/10.6; and sialic acid, 14.2/13.0. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot identified a homogeneous 68–70 kDa molecular species for normal and acute-phase thiostatin. Inter-sugar linkage analysis was carried out for permethylated oligosaccharides released by hydrazinolysis. Gas chromatography yielded the following partially methylated alditol acetates relative to 1.0 mole of 1,3,6-tri-*O*-linked mannose (mean normal/mean acute phase): galactose: 1,3-di-*O*-, 1.44/1.01; 1,6-di-*O*-, 1.02/0.68; mannose: 1,2-di-*O*-, 1.64/1.42; 1,2,4-tri-*O*-, 0.24/0.13; 1,3,6-tri-*O*-, 1.0/1.0; 2-deoxy-2-*N*-methylacetamidoglucose: 1,4-di-*O*-, 1.42/1.12. These analytical studies indicated that corresponding carbohydrate structures are present in normal and acute-phase thiostatin. Crossed affinoimmunoelectrophoresis (CAIE) further confirmed the structural similarity between the glycan moieties.

Keywords: acute phase, cysteine proteinase inhibitor, glycosylation, kininogen, thiostatin

Introduction

Rat plasma thiostatin is a 68 kDa glycoprotein member of the mammalian cysteine proteinase inhibitor (CPI) superfamily [1]. Thiostatin is composed of a heavy chain (~45 kDa), a light chain (~4 kDa) and an internal kinin segment (~1 kDa). The cDNA sequence of thiostatin contains five putative asparagine-linked glycosylation sites [2]. CPI sites reside in the cystatin-like domains of the heavy chain. We have shown that chemical deglycosylation abolishes the CPI activity of thiostatin [3].

Thiostatin is also a low molecular weight kininogen substrate component of the kallikrein–kininogen–kinin (K–K–K) protease cascade system. The kininogens ordinarily are hydrolyzed by serine protease kallikreins to release bioactive peptide kinins, most notably bradykinin (reviewed in ref. 4). Rat plasma uniquely contains the 'kallikrein-resistant' low molecular weight kininogen,

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thiostatin. We originally isolated and purified this protein and found it susceptible to cleavage by a rodent tumor acid protease to yield a novel Ile-Ser-bradykinin vasopeptide [5, 6]. Although thiostatin itself has been found only in the rat, Ile-Ser-bradykinin has been identified in the ascites fluid of a patient with ovarian carcinoma [7].

In addition to its CPI and kinin donor functions, thiostatin belongs to the acute-phase plasma protein (APPP) family. The acute-phase response (APR), a generalized organismal reaction, is elicited by the trauma of tissue injury, the sources of which include infection, neoplasia, noxious chemicals and immune system disorders (reviewed in ref. 8). The APR is characterized by dramatic alterations in the synthesis of several APPPs. In the APR of the rat, thiostatin is the only kininogen with an elevated expression [9].

The majority of APPPs are glycoproteins. The structure of the glycan moieties is known to be modified for some APPPs as a result of acute-phase inflammatory stimuli [10]. Neither the mechanism nor the biological importance of the altered glycosylation is understood. This study compares the carbohydrate structure of thiostatin purified from normal and acute-phase 'thiostatin-enriched' rat plasma. Subcutaneous injection of a small volume of turpentine was used in our study to precipitate the APR [11]. Normal and acute-phase thiostatin were compared on the basis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blot and sugar compositional analysis. Inter-sugar linkage analysis was performed on oligosaccharides released by hydrazinolysis. Both proteins also were subjected to crossed affinoimmunoelectrophoresis (CAIE) with concanavalin A as affinity component in the first dimension and polyclonal antisera in the second dimension.

Materials and methods

N-acetylneuraminic acid, D-arabinose, ethylene glycol, sodium borohydride, α-methyl-D-glucoside and acetic anhydride all were purchased from Sigma (St Louis, MO, USA). Concanavalin A was obtained from Calbiochem (San Diego, CA, USA). Sephadex G-25 was purchased from Pharmacia (Piscataway, NJ, USA). Dimethyl sulphoxide, methanol, chloroform and pyridine were from J.T. Baker (Phillipsburg, NJ, USA). Bio-Gel P2, AG1-X2 and AG50W-X4 were obtained from Bio-Rad (Richmond, CA, USA). Sodium hydride and methyl iodide were purchased from Aldrich (Milwaukee, WI, USA). Anhydrous hydrazine was obtained from Pierce (Rockford, IL, USA). Partially methylated alditol acetate standards were prepared as described below with sugars purchased from Sigma. Dura-Bond (DB) capillary gas chromatography columns DB-1, DB-17 and DB-225 were products from J & W Scientific (Folsom, CA, USA).

Purification of thiostatin

Thiostatin was purified by passage of normal and acute-phase rat plasma through a carboxymethyl-papain Sepharose 4B affinity chromatography column as described previously [12].

Sugar compositional analysis

Individual neutral sugars were analyzed as their alditol acetates following hydrolysis with 2 N HCl at 100°C for 4 h. Processing of samples and gas chromatography conditions have been described previously [3]. Sialic acid content was determined by the thiobarbituric acid assay of Warren [13].

SDS–PAGE and Western blot

Electrophoresis was carried out on a 10% polyacrylamide gel under reducing and denaturing conditions [14]. Western blot [15] of normal and acute-phase thiostatin electrophoresed under the same conditions utilized antiserum prepared previously [5].

Inter-sugar linkage analysis of oligosaccharides

Preparation of oligosaccharides by hydrazinolysis. Liberation of *N*-linked oligosaccharides was accomplished by hydrazinolysis [16]. Briefly, thiostatin (50 mg) was incubated with 5.0 ml of anhydrous hydrazine for 16 h at 100°C, dried under a stream of nitrogen and desalted on a Bio-Gel P2 column (1.0 × 40 cm) equilibrated with 0.1 M pyridine acetate, pH 5.0. Carbohydrate-containing fractions, identified by the phenol sulfuric acid method [17], were pooled, lyophilized, re-*N*-acetylated and desalted on a Sephadex G-25 column (1.5 × 95 cm) equilibrated with 0.5% ammonium bicarbonate, pH 8.5. Carbohydrate-positive fractions were pooled, lyophilized and reduced with 1 ml of a sodium borohydride solution (10 mg/ml 0.05 M NaOH) for 16 h at 20°C. Samples were dried under vacuum in the presence of toluene and purified on a Bio-Gel P2 column (1.0 × 40 cm) equilibrated with 0.1 M pyridine

acetate, pH 5.0. Carbohydrate-containing fractions were pooled, lyophilized, dissolved in 1.0 ml of water and stored at −20°C.

Methylation of oligosaccharides. Partially methylated alditol acetates (PMAAs) were prepared from thiostatin according to the method of Anumula & Taylor [18]. Briefly, 100 μg of neutral sugar equivalents obtained by hydrazinolysis was permethylated in a round-bottom tube (13 × 100 mm) containing dimethylsulfoxide, dimsyl sodium ion and methyl iodide. Carbohydrate was extracted with chloroform, washed four times with water, the organic layer dried under nitrogen and the permethylated sugars hydrolyzed with formic acid and trifluoroacetic acid. Dried samples were reduced with sodium borohydride, acetylated with acetic anhydride and extracted with chloroform. PMAAs were dried in preparation for gas chromatography.

Gas chromatography. PMAAs were dissolved in an appropriate volume of chloroform for injection into a gas chromatograph, the Varian Series 3400 instrument equipped with a flame ionization detector, and connected to a Varian 4290 integrator. Samples were resolved by a DB-17 capillary column (15 m long, 0.519 mm i.d., 1.0 μm phase thickness). The carrier gas was nitrogen. Injections were performed in the splitless mode. The column program began with an isocratic hold at 140°C for 10 min followed by a linear gradient to 200°C (2°C/min) held for 5 min and finally a linear gradient up to 225°C (5°C/min) maintained at that temperature for 5 min.

PMAAs derived from the oligosaccharides were identified and quantitated based on their retention times relative to those of PMAAs prepared from sugar standards. The identity of permethylated sugar standards was unequivocally determined by mass fragmentographs obtained from gas chromatography–mass spectroscopy (GC–MS), described below. Permethylated sugar standards were prepared from the following: (1) methyl 3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside, (2) methyl 4-*O*-(3-*O*-[2-acetamido-2-deoxy-4-*O*-β-D-galacto pyranosyl-β-D-glucopyranosyl]-β-D-galactopyranosyl) β-D-glucopyranoside, (3) 2-acetamido-2-deoxy-4-*O*([4 - *O* - β - D - galactopyranosyl] - β - D - glucopyranosyl) - D - glucopyranose, (4) methyl-2-*O*-α-D-mannopyranosyl-α-D-mannopyranoside, (5) benzyl-2 - acetamido - 2 - deoxy - α - D - galactopyranoside and (6) 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucopyranose. Each standard sugar was prepared separately as a 1 mg/ml solution in high-performance liquid chromatography (HPLC)-grade water and 200-μl aliquots (200 μg) taken for permethylation. These standard PMAAs were analyzed on a Finnegan 4500 GC–MS system operating in the electron impact mode. A DB-1 capillary column was used to resolve the PMAAs (30 m long, 0.25 mm i.d., 0.25 μm phase thickness). The carrier was helium (19 p.s.i., 38 cm/s). Injections were performed in the splitless mode. The column program began with an isocratic hold at 80°C (2 min), followed by a linear gradient to 150°C (10°C/min) and a final linear gradient to 280°C (2°C/min); the 280°C temperature was maintained for 10 mins. Temperatures of the ion source, injector and transfer lines were 180°C, 250°C and 290°C respectively. A scan time of 1.5 s/scan was used and the INCOS Data System facilitated data handling.

Crossed affinoimmunoelectrophoresis (CAIE)

CAIE was performed in accord with the method of Bog-Hansen *et al.* [19]. The first-dimension gel contained concanavalin A (Con A) at a concentration of 1.0 mg/ml 1% agarose and the second-dimension gel, also made with 1% agarose, contained 1% monospecific rabbit anti-rat thiostatin antiserum [5]. In addition, the second-dimension gel included α-methyl-D-glucopyranoside (40 mg/ml). Electrophoresis was run at 10 V/cm for 1.5 h in the first dimension and 6 h in the second dimension. The apparatus was cooled with a circulating water bath.

Results

Sugar composition of normal and acute-phase thiostatin

Table 1 lists the carbohydrate composition for thiostatin purified from normal versus acute-phase rat plasma. Highly similar mean estimates, on a mole per mole basis, were determined for each component of normal (N) and acute-phase (AP) thiostatin: fucose, 2.4 (N), 1.7 (AP); mannose, 7.5 (N), 8.0 (AP); galactose, 11.2 (N), 10.6 (AP); sialic acid, 14.2 (N), 13.0 (AP).

Comparative SDS–PAGE and Western blot of normal versus acute-phase thiostatin

As the primary sequence of both normal and acute-phase thiostatin is nearly identical, a significant difference in gel migration would suggest distinct carbohydrate structures. Thiostatin samples

Table 1. Carbohydrate composition of thiostatin from normal and acute-phase rat plasma

Sugar	Thiostatin (mol/mol)[a]	
	Normal	Acute phase
Fucose	2.0	1.2
	2.4	2.0
	2.9	1.8
Mannose	8.7	7.8
	6.0	6.6
	7.7	9.5
Galactose	13.1	12.4
	12.2	9.0
	8.2	10.3
Sialic acid	13.5	12.7
	14.7	12.9
	14.3	13.4

[a]Each neutral sugar was determined by gas chromatography of alditol acetates derived from thiostatin as described under 'Sugar compositional analysis' in the Materials and methods section. Sialic acid was determined by the method of Warren [13].
One preparation of normal and acute-phase thiostatin was used. Three determinations are reported for the neural sugar analysis. Three separate aliquots were used in one sialic acid assay.

were electrophoresed on 10% acrylamide under reducing and denaturing conditions (Figure 1). Both normal (lane B) and acute-phase (lane C) samples appeared as homogeneous protein bands of 68 kDa.

The Western blot shown in Figure 2 reveals a single band of approximately 70 kDa for normal (lane B) and acute-phase (lane C) thiostatin, thereby confirming the homogeneity and similar size of both immunoreactive species.

Inter-sugar linkage analysis of the oligosaccharides from normal and acute-phase thiostatin

The inter-sugar linkage pattern was compared for oligosaccharides released by hydrazinolysis of normal and acute-phase thiostatin. Oligosaccharides were permethylated to yield partially methylated alditol acetates (PMAAs) analyzed by gas chromatography. Table 2 reports the molar amount for each PMAA derived from normal (N) and acute-phase (AP) thiostatin. Based on an assigned value of 1.0 to 1,3,6-linked mannose (core mannose), the following mean quantities were determined for the variously substituted residues: galactose: 1,3-linked, 1.44 residues (N) compared with 1.01 residues (AP); 1,6-linked, 1.02 residues (N) versus 0.68 residues (AP); mannose: 1,2-linked, 1.64 residues (N) compared with 1.42 residues (AP); 1,2,4-linked, 0.24 residues (N) compared with 0.13 residues (AP); 2-deoxy-2-*N*-methylacetamidoglucose: 1,4-linked, 1.42 residues (N) and 1.12 residues (AP). These data indicate similar carbohydrate structures for both proteins. The molar ratios for the major peaks of mannose and galactose manifest the presence of biantennary

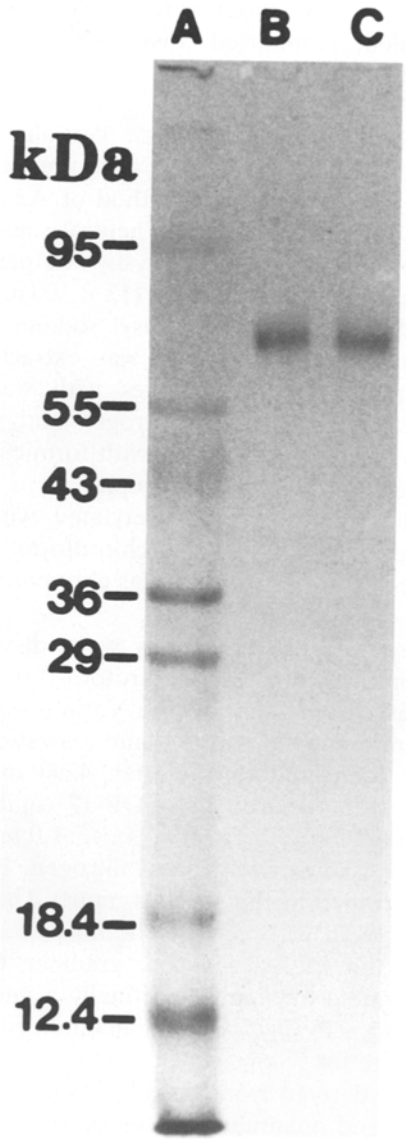

Figure 1. SDS–PAGE of thiostatin from normal and acute-phase rat plasma. Molecular weight marker proteins (lane A), 1 μg each of normal (lane B) and acute-phase (lane C) thiostatin were electrophoresed on a 10% polyacrylamide gel under reducing and denaturing conditions. The apparent molecular weight of both proteins was 68 kDa.

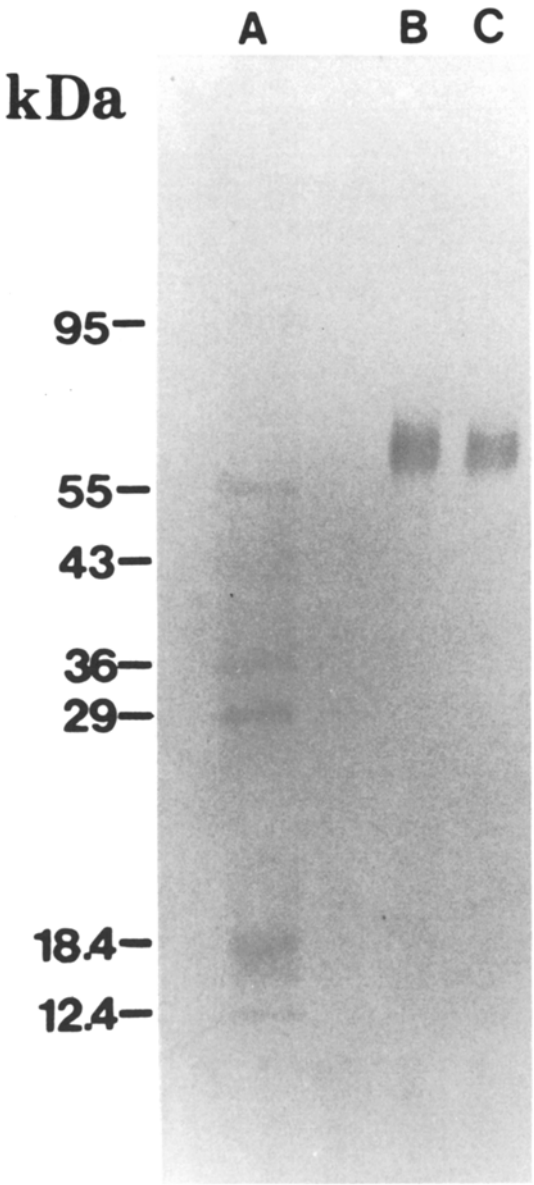

Figure 2. Western blot of normal and acute-phase rat plasma thiostatin. Thiostatin (0.5 μg) was resolved by SDS–PAGE (10% gel) and detected with rabbit anti-rat thiostatin antiserum (1:4000 dilution). Lanes A, B and C represent marker proteins, normal thiostatin and acute-phase thiostatin respectively. The molecular weight of the thiostatin was determined as approximately 70 kDa.

complex glycan. The minor peak of 1,2,4-substituted mannose reveals the presence of a smaller amount of triantennary structure. Approximately 4–5 residues of 1,4-linked *N*-acetylhexosamine are predicted for bi- and triantennary structures. The identification of only approximately 1–1.5 residues of 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucitol reflects the poor recovery commonly observed for permethylated hexosamines [16].

Crossed affinoimmunoelectrophoresis (CAIE) of normal and acute-phase thiostatin

Purified normal and acute-phase thiostatin resolved by concanavalin A (Con A) reactivity (first dimension) was immunoprecipitated with monospecific antiserum (second dimension) (Figure 3). Normal thiostatin (Figure 3a) yielded a slower migrating major peak in the first dimension; a 'camel' shape suggests glycan heterogeneity possibly due to fucosylation or sialylation. A smaller, less reactive peak migrated at a faster rate in the first dimension. The area under each component was estimated by the product of the peak height and peak width at half-height. Based on the known reactivity of the various glycan branching patterns with Con A [20], and the relative area of the different peaks, the less reactive minor component, which represents about 30% of the total peak area ($28 \pm 3\%$ based on three determinations), corresponds to the minor amount of triantennary structure detected by methylation analysis (0.24 residues of 1,2,4-trisubstituted mannose per core mannose); the major, slower migrating fraction represents biantennary glycan.

The CAIE profile for acute-phase thiostatin shown in Figure 3b is essentially that displayed by normal thiostatin (Figure 3a), supporting the similarity of carbohydrate structures on both proteins. The faster migrating peak constituted approximately 20% ($22 \pm 5\%$) of the total peak area, in agreement with inter-sugar linkage analysis (0.13 residues of 1,2,4-trisubstituted mannose per residue of core mannose).

Discussion

Thiostatin was utilized as a model glycoprotein to study the carbohydrate structure of an APPP elaborated during an inflammatory response. The altered hepatic synthesis of APPPs is mediated by cytokines, notably interleukin 1 (IL-1), IL-6 and tumor necrosis factor-α [8]. In addition to altered plasma levels of APPPs during the APR, acute inflammation has been implicated in altered glycosylation patterns of APPPs. Under inflammatory conditions human α_1-proteinase inhibitor, ceruloplasmin [10] and rat and human α_1-acid glycoprotein [21] display shifts in their Con A reactivity profiles, indicative of increased levels of biantennary glycan. On the other hand, increased levels of

Table 2. Comparative molar ratios of partially methylated alditol acetates obtained from normal and acute-phase thiostatin oligosaccharides (values determined on a DB-17 column)[a]

Methylated sugar	Position substituted	Molar ratio[b]	
		Normal	Acute phase
Galactose			
2,4,6-Tri-*O*-methyl	1,3-Di-*O*-	1.46	1.03
		1.41	0.98
2,3,4-Tri-*O*-methyl	1,6-Di-*O*-	1.08	0.68
		0.95	0.68
Mannose			
3,4,6-Tri-*O*-methyl	1,2-Di-*O*-	1.76	1.46
		1.53	1.38
3,6-Di-*O*-methyl	1,2,4-Tri-*O*-	0.30	0.12
		0.17	0.14
2,4-Di-*O*-methyl	1,3,6-Tri-*O*-	1.0	1.0
2-Deoxy-2-*N*-methylacetamidoglucose			
3,6-Di-*O*-methyl	1,4-Di-*O*-	1.60	0.98
		1.25	1.26

[a]Gas chromatography conditions are described in the Materials and methods section.
[b]Assume 1,3,6-tri-*O*-mannose = 1.0.
One preparation was used for normal thiostatin and two separate preparations for acute-phase thiostatin. Two determinations are reported for normal thiostatin and one determination for each preparation of acute-phase thiostatin.

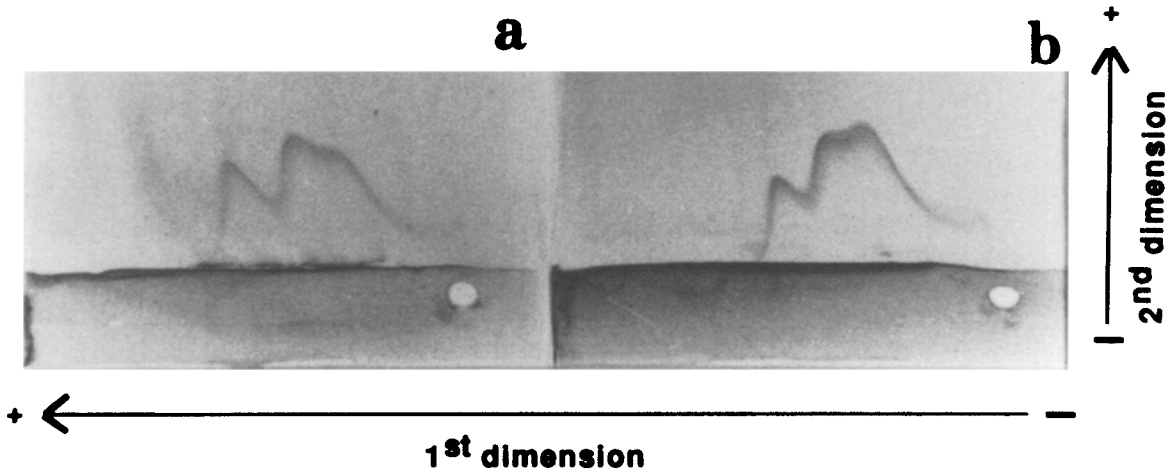

Figure 3. Crossed affino-(Con A)-immunoelectrophoresis (CAIE) of normal and acute-phase thiostatin. Normal thiostatin (a) and acute-phase thiostatin (b) were resolved in the first dimension on a 1% agarose gel containing 1 mg/ml Con A and in the second dimension on a 1% agarose gel containing 1% rabbit anti-rat thiostatin monospecific antiserum and 40 mg/ml α-methyl-D-glucopyranoside. In both a and b, two major components were observed: (a) predominant slower migrating peak with a 'camel' appearance and a smaller peak which migrated further in the first dimension.

triantennary glycan have been identified for α_1-proteinase inhibitor from patients with acute inflammation [22]. Such modifications in the glycan units of APPPs have evoked questions regarding the biological role of these structures in the context of inflammation. An interesting report concerns the possible role of the glycan units of one APPP, the human α_1-acid glycoprotein (AGP). AGP consists of different glycoforms containing biantennary, triantennary and tetra-antennary units. AGP containing none, one or two biantennary glycan units is identified as AGP-A, B

or C respectively. The remainder of the glycans on these forms are tri- and tetra-antennary. Carpentier *et al.* [23] have demonstrated that the efficiency of binding and internalization of AGP-C to human monocytic lineage cells correlates with differentiation state, increasing from promonocytic cells to mature macrophages. The authors speculate that AGP-C may serve a role in immunoregulation based on an inflammation-induced increase in the relative amount of this glycoform [24].

This study compared the carbohydrate structures of normal and acute-phase thiostatin. Compositional analysis of the neutral sugar and sialic acid content of both proteins yielded nearly identical results, indicative of similar structures (Table 1). Thiostatin purified from both normal and acute-phase plasma migrated the same distance in SDS–PAGE (Figure 1), and immunoreactive protein was identified as a single band of approximately 70 kDa (Figure 2), also signifying similar carbohydrate structures.

Inter-sugar linkage analysis was carried out for the *N*-linked oligosaccharides liberated by hydrazinolysis of normal and acute-phase thiostatin. Gas chromatography of the permethylated samples revealed the same major peaks for both proteins (Table 2). The molar amount relative to core mannose (1,3,6-mannose) was similar for each corresponding peak. The major *N*-linked carbohydrate structure of thiostatin, whether expressed under normal conditions or during acute inflammation, consists of biantennary glycan and a lesser amount of triantennary glycan.

Further support for highly similar carbohydrate structures was provided by CAIE, an electrophoretic technique that can resolve normal and acute-phase thiostatin by Con A affinity (first dimension) and immunoprecipitation with monospecific anti-serum (second dimension). The relative amount of different glycan branching patterns was estimated by the CAIE profiles shown in Figure 3a & b. An overall similarity for both proteins was apparent, with two major components observed, including a predominant, more highly reactive fraction with a 'camel' appearance and a minor, less reactive, peak. Calculation of the area under the curve for each peak yielded estimates of bi- and triantennary glycan consistent with inter-sugar linkage analysis.

Baussant *et al.* [25] also have identified a biantennary branching pattern for the *N*-linked oligosaccharides of thiostatin purified from normal and inflamed rats. Employing different methodology they were able to elucidate further the fine carbohydrate structure. Thiostatin purified from normal and acute-phase plasma was resolved into reactive and non-reactive fractions by Con A affinity column chromatography and oligosaccharides were liberated by hydrazinolysis and purified by HPLC for subsequent analysis. Proton-nuclear magnetic resonance (^{1}H-NMR) spectroscopy identified the presence of disialylated and trisialylated biantennary glycan for normal thiostatin and a significantly decreased amount of trisialylated glycan for inflamed thiostatin.

In summary, inflammation-induced protein glycosylation can result in new levels of either biantennary or triantennary glycan for some APPPs. However, as in the case of thiostatin, the carbohydrate structure of other glycoproteins may remain essentially the same. Therefore, the mechanisms of post-translational modification which operate in response to inflammation need to be elucidated in order to explain why proteins are glycosylated in unique ways during the acute-phase

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