of the Laboratory of Medical Entomology, Kaiser Foundation Research Institute, for his interest and encouragement in this work. Dr. John M. Stewart of the Department of Biochemistry, University of Colorado, is thanked for his continuing advice on the chemical aspects of this study. We also wish to thank Drs. J. R. Kettman, D. Michaeli, W. J. Peterson, and Mr. M. Shimizu for their valuable advice and discussions. We especially thank Mrs. S. San Juan for performing the immunological assays and Mr. M. Shimizu for the photographs of the peptide models.

References

Benjamini, E., Shimizu, M., Young, J. D., and Leung, C. Y. (1968a), *Biochemistry 7*, 1253.

Benjamini, E., Shimizu, M., Young, J. D., and Leung, C. Y. (1968b), *Biochemistry 7*, 1261.

Benjamini, E., Young, J. D., Peterson, W. J., Leung, C. Y., and Shimizu, M. (1965), *Biochemistry 4*, 2081.

Funatsu, G. (1964), *Biochemistry 3*, 1951.

Merrifield, R. B. (1964), *Biochemistry 3*, 1385.

Stewart, J. M., Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1966), *Biochemistry 5*, 3396.

Tsugita, A., Gish, D. T., Young, J. D., Fraenkel-Conrat, H., Knight, C. A., and Stanley, W. M. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1463.

Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1966), *Biochemistry 5*, 1481.

Young, J. D., Benjamini, E., Shimizu, M., and Leung, C. Y. (1967b), *Intern. Congr. Biochem., Tokyo, IV*, 693.

Young, J. D., Benjamini, E., Stewart, J. M., and Leung, C. Y. (1967a), *Biochemistry 6*, 1455.

Glycopeptides from Fibrinogen and Fibrin*

Bonnie Anderson Bray† and K. Laki

ABSTRACT: Four major glycopeptides fractions were isolated from bovine fibrinogen and from fibrin. Two of them are characterized by the presence of lysine and the other two by the presence of arginine, glutamic acid, glycine, and valine. One of the lysine-containing glycopeptides present in both fibrinogen and fibrin contained 1.4–2.0 moles of sialic acid. This glycopeptide has not been reported previously. (When this manuscript was in preparation, we read the report of Mester *et al.* (Mester, L., Moczar, E., and Szabados, L. (1967), *Compt. Rend. Acad. Sci. Paris 265*, 877) who have now obtained as many as seven glycopeptides by high-voltage electrophoresis, one of which has lysine and 24% sialic acid.) These data and the facts that in the glycopeptide mixtures arginine and lysine are equal on a molar basis, and that glutamic acid is present to the extent of only 3 moles/mole of fibrinogen indicate the chains of fibrinogen are not all paired with respect to their glycopeptides. Degradation of one of the glycopeptides with sialidase followed by crude β-glucosidase indicated the sequence from the nonreducing end of sialic acid, galactose, *N*-acetylglucosamine, and mannose.

In each glycopeptide, both *N*-acetyl- and *N*-glycolylsialic acids were present and could be removed by purified *Clostridium perfringens* sialidase. The glycopeptides from cross-bonded fibrin contained 1 mole less of aspartic acid than those from fibrinogen. Also, one of the glycopeptides from fibrin was held more strongly on Dowex 1 (formate column) than was its counterpart from fibrinogen.

The carbohydrate moiety of fibrinogen (Laki, 1951a) may be necessary to the function of fibrinogen as a substrate for thrombin since periodate oxidation of bovine fibrinogen results in a 50% loss of clottability by thrombin when 30% of the hexose and 40% of the sialic acid were oxidized (Laki and Mester, 1962). There is a controversy as to whether the carbohydrate is involved when fibrin is stabilized by the enzyme variously called Laki–Lorand factor, fibrinase, or factor XIII. Enzymatic removal of the sialic acid resulted in a fibrinogen which was not capable of stabilization by the enzyme (Laki and Chandrasekhar, 1963), but on the question of loss of sialic acid under the action of the Laki–Lorand factor, there are reports pro (Chandrasekhar *et al.*, 1962, 1964; Chandrasekhar and Laki, 1964) and con (Blombäck, 1958; Raisys *et al.*, 1966). The same confusion exists regarding the possibility that stabilized fibrin contains 10–20% less hexose, there being reports pro (Laki, 1951a; Blombäck, 1958; Brown, 1963; Szara and Bagdy, 1953; Bagdy and Szara, 1955) and con

* From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received March 22, 1968.* A preliminary account of this work has already appeared (Laki, 1968).

† Present address: New York Blood Center, New York, N. Y. 10021.

(Rosenberg and Carman, 1964; Tyler, 1966; Hörmann and Gollwitzer, 1964). This situation is not surprising in view of the technical difficulties involved. It appeared important, therefore, to study the role of the carbohydrate moiety of fibrinogen and fibrin preparations in which the carbohydrate content is enriched by digesting away the protein component.

Mester *et al.* (1963a,b, 1965a,b), Cynkin and Haschemeyer (1964), and Haschemeyer *et al.* (1966) have studied the glycopeptides obtained by Pronase digestion of bovine fibrinogen and fibrin. Mester's group worked with sialidoglycopeptides separated by electrophoresis; the other workers removed sialic acid to expedite Pronase digestion. In the present study on bovine material, we have compared the sialidoglycopeptides obtained by Pronase digestion from fibrinogen with those from fibrin stabilized with a transglutaminase isolated from guinea pig liver (Folk and Cole, 1966) which was shown by Tyler and Laki (1966) to be capable of cross-linking fibrin. The sialidoglycopeptides before and after separation on Dowex 1 were analyzed for their carbohydrate and amino acid contents. From the data the conclusion was reached that the chains of fibrinogen are not all paired with respect to the distribution of their carbohydrate moiety. Also, there were indications that a carbohydrate-carrying asparagine residue is involved in the cross-linking process.

Materials and Methods

Fibrinogen and Fibrin. Three separate preparations, one of which was converted into stabilized fibrin, were made from the same lot (B-8209) of bovine fibrinogen from Armour Pharmaceutical Co., Kankakee, Ill. The fibrinogen (50 g containing 40–60% sodium citrate) was purified by the procedure of Laki (1951b) and the resulting protein (~15 g) was 96–99% clottable. Preparations to be used as fibrinogen were not dialyzed *vs.* KCl but after the ammonium sulfate precipitation, they were dissolved in water to a final concentration of 5% (the residual ammonium sulfate facilitated the solution). For preparation of fibrin, the fibrinogen was dialyzed in the cold against 8 l. of 0.3 M KCl (pH 7) for 2.5 days. To the final volume of 300 ml containing 15 g of fibrinogen was added 100 ml of 0.1 M $CaCl_2$ and water to 950 ml. In a 3-l. beaker was placed 1.5 ml of thrombin (1500 units, topical bovine, Parke Davis) and 8 ml of guinea pig liver transglutaminase (Folk and Cole, 1966). The fibrinogen mixture was added to the enzyme mixture quickly with stirring. Two samples were removed for testing the stability of the fibrin and allowed to incubate for 15 min and for 1 hr, respectively, at room temperature before an equal volume of 8 M urea was added to the tube. After 1 hr the clot was stabilized; *i.e.*, it did not dissolve after being in 4 M urea for 1 hr. The main body of the clot after 1 hr at room temperature was allowed to incubate overnight in the cold room. The clot was squeezed out by hand and washed once with 0.15 M KCl. The supernatant and washes were lyophilized. The clot was cut into small pieces and suspended in 300 ml of water for the Pronase digestion.

Preparation of Glycopeptides. These procedures are essentially those used by Mester *et al.* (1963a). During all steps except enzymatic digestion, solutions were kept cold and preparations were stored frozen until processed and then were lyophilized and stored in a refrigerated desiccator. These precautions were observed to prevent hydrolysis of sialic acid.

Solutions of fibrinogen or the suspension of stabilized fibrin, all at ~5% concentration, were neutralized to pH 7 with 1 N NaOH. Solid Pronase (Calbiochem) in an amount of 24 mg/g of substrate was added. Digestions were carried out at 37°; samples were layered over with toluene to prevent bacterial growth. The progress of the reaction was followed by the ninhydrin test (Moore and Stein, 1948) using leucine as a standard. After 30 hr, fibrinogen preparation I gave 18.5% leucine value. (This means roughly that on the average every fifth peptide bond is broken.) Fibrinogen preparation II was digested the same way for 26 hr. Then an amount of Pronase equal to 3 mg/g of substrate and $CaCl_2$ to a final concentration of 0.01 M were added and the digestion was continued for an additional 19 hr. Pronase (3 mg/g of substrate) was added again and the mixture was digested for 16 hr. At this point the digestion had leveled off at 32.4% leucine value. The fibrin suspension was more readily digested by Pronase than were the fibrinogen preparations. At the end of a single 30-hr digestion, it had leveled off at 50% leucine value.

Purification of Glycopeptide Mixtures. In the case of fibrinogen preparation I the thawed digest was centrifuged to remove free tyrosine which had come out of solution. The supernatant was concentrated on a flash evaporator to 160 ml. Four volumes (640 ml) of absolute ethanol were added and the suspension was left in the refrigerator overnight. An oily precipitate was obtained by centrifugation; it was dissolved in water and lyophilized (17.1 g). In each of two runs half of this material was dissolved in 0.1 M acetic acid (50 ml including rinses) and passed through a Sephadex G-25 column equilibrated with 0.1 N acetic acid. Fine resin (20–70-μ particle size) was used in a resin bed of 105 × 5 cm dimensions. The column was run at 102 ml/hr and 17-ml fractions were collected on a Spinco fraction collector. At least 97% of the hexose was present in a symmetrical peak in the void volume. The large hexose-containing peaks from both runs were combined and lyophilized. Electrophoresis revealed the presence of many ninhydrin-positive bands and the mixture was further purified by trichloroacetic acid precipitation in the cold. To a 50-mg/ml solution in water of the glycopeptides was added an equal volume of 15% trichloroacetic acid. After mixing, the precipitate was removed by centrifugation. The trichloroacetic acid was removed from the supernatant by extraction with ether whereupon the material was lyophilized.

In the case of fibrinogen preparation II and fibrin, the trichloroacetic acid step was performed on the glycopeptides obtained by alcohol precipitation after extracting the water-soluble material into 75 ml of water. The lyophilized trichloroacetic acid supernatant could then be passed through the Sephadex column in one operation.

Resolution of Glycopeptide Mixtures. Dowex 1 (AG

1-X2, 100–200 mesh, Cl^- form from Bio-Rad) was suspended in 3 N sodium formate overnight and washed in a fitted glass funnel sequentially with 3 N sodium formate, water, 91% formic acid, and water, whereupon Cl^- was barely detectable. The resin was suspended in 1 N sodium formate; the column (0.9 × 70 cm resin bed) was packed and washed with 1 N sodium formate until no Cl^- was detectable in the effluent and then with water. Glycopeptide mixtures (fibrinogen I, 454 mg; fibrinogen II, 350 mg; and fibrin, 353 mg) were applied in 2.5 ml of water including rinses. The column was eluted at 12 ml/hr first with 100 ml of water (0.5 ml/tube) and then with a linear formate gradient at pH 5.0 (2 ml/tube). Molar formic acid adjusted to pH 5.0 with pyridine was drawn into the water in the mixing vessel by a connection at the bottom of the two vessels which were of equal size. From the mixing vessel, which was stirred magnetically, the solution was siphoned into the top of the column. Fractions were screened by the Loewus (1952) anthrone method.

Electrophoresis was carried out on 6-in. wide strips of Sepraphore III in a Gelman deluxe electrophoresis chamber at pH 6.4 in a pyridine–acetic acid buffer at 13 V/cm for 3 hr. The top of the cell was cooled with an ice pack. The use of the periodic acid–Schiff stain had to be modified because the glycopeptides are soluble in trichloroacetic acid and would not be fixed to the Sepraphore as a glycoprotein would be. The strip was dried while lying flat on a smooth surface. It was then taped to a frame and sprayed to a dull even dampness with 0.5% periodic acid. It was allowed to dry *in situ* to a chalky whiteness. Then it was sprayed to a dull even dampness with the dye solution. The pink bands appeared after 5–10 min. When dry again, the strip was washed repeatedly with absolute methanol and was cleared by leaving it 75 sec in a 10% (v/v) solution of glacial acetic acid in methanol and removing the strip and laying it smoothly on a glass plate.

Analyses. The fibrinogen preparations were analyzed for protein content by the Biuret method.

For hexoses the anthrone method of Loewus (1952) or of Dreywood (1946) and the phenol–sulfuric acid method (Dubois *et al.*, 1956) were used. Sialic acid was determined by the Warren (1959) method after sealed-tube hydrolysis for 1 hr at 100° in 0.05 M citrate buffer (pH 3.05). The manual procedure of Swann and Balazs (1966) was the modification of the Elson–Morgan (1933) reaction used for hexosamines. Samples were hydrolyzed in a sealed tube at 1 mg/ml in 4 N HCl for 16 hr at 100°; an hydrolysis time curve on glycopeptide 3 showed a plateau from 6 to 24 hr under these conditions. Amino acids and sugars found in the two types of glycopeptides (one containing lysine, the other arginine) were added to the standards in the concentrations present in the glycopeptides. No interference was seen in the color test.

N-Acetylhexosamine was determined by the Reissig–Strominger–Leloir (1955) test. The automated procedure of Spackman *et al.* (1958) was used for amino acid analyses on glycopeptides after hydrolysis *in vacuo* in 2 ml of constant-boiling HCl for 22 hr at 110°.

Enzymatic Degradation of the Glycopeptides. Sialic acid was removed with the purified *Clostridium perfringens* enzyme from Worthington Biochemical Corp. (Cassidy *et al.*, 1966) at pH 4.37 in 0.1 M acetate. In the case of glycopeptide 15 the removal of *N*-glycolylsialic acid occurred only when the digestion mixture was 0.02 M in EDTA. Samples were chromatographed on thin-layer chromatography plates precoated with cellulose F (E. Merck, Darmstadt, Germany, Brinkmann) in ethyl alcohol–water–ammonia (80:20:1) (Chandrasekhar *et al.*, 1966). Sialic acids were detected by the Warren (1960) method. Standard *N*-acetylneuraminic acid synthetic, 99% pure, was obtained from General Biochemicals and *N*-glycolylneuraminic acid from porcine submaxillary glands, crystalline, type II, was obtained from Sigma Chemical Co.

Digestion with crude β-glucosidase (EC 6104-9 from Worthington Biochemical Corp.) was carried out to see what the sequence of sugars was from the nonreducing end of the glycopeptide. The glycopeptide (4 mg) was dissolved in 0.4 ml of 0.1 M acetate buffer (pH 5.0). To 0.2 ml of the solution was added 0.5 mg of purified *C. perfringens* sialidase. It was incubated under toluene at 37° for 24 hr at which time free sialic acid was detected by spraying a sample dried onto paper. To the sialidase-treated and the untreated glycopeptide 4 were added 1.9 and 2.2 mg, respectively, of the crude glucosidase. From the sialidase-treated sample, 5 μl was spotted onto a thin-layer chromatography plate and 10 μl was taken into a test tube for *N*-acetylhexosamine analysis at various times. The glycopeptide treated only with glucosidase was also sampled at the same times but 10 μl was spotted onto the plate and 20 μl was taken for *N*-acetylhexosamine. They were incubated under toluene at 37–42° and sampled at zero time, 10, 58, 72, 120, 216, and 240 hr. This plate was run in Fisher–Dörfel (1955) solvent and sprayed with aniline hydrogen phthalate (Partridge, 1949).

Approximate molecular weights were calculated from experiments made in the Spinco Model L analytical ultracentrifuge using the artificial boundary cell and a value of 0.664 for partial specific volume, which has been determined experimentally for similar glycopeptides from fetuin (Spiro, 1962).

Results

Sialic acid was used as a basis for estimating total recovery of carbohydrate in the glycopeptide mixtures because this analysis is less subject to the large interferences due to protein that plaque the analyses for hexose and hexosamine. The problem with using the sialic acid analysis as a basis is the ease with which the sialic acid is hydrolyzed. However, in all three products the recovery (milligrams of sialic acid per gram of fibrinogen) was the same (Table I). In the two instances where the fibrinogen was analyzed beforehand (fibrin and fibrinogen preparation II), the recoveries based on sialic acid were 69 and 70%, respectively. When the recoveries were corrected up to 100% in order to compare the glycopeptides on a molar basis (Table II), reasonable figures were obtained; *i.e.*, 5.6 moles of glycopeptide/mole of fibrinogen. The data of Hen-

TABLE I: Glycopeptide Mixtures and Sialic Acid Recovery.

	Starting Material		Glycopeptide Mixtures				
				Sialic Acid			
	g	Sialic Acid (mg)	mg	%	mg	% Recov	Fibrinogen (mg/g)
Fibrinogen I	18		795[a]	8.5	67.6		3.8
II	14.2	79.6	407	13.6	55.5	70	3.9
Fibrin	15	81	406	13.7	55.6	69	3.7

[a] Before trichloroacetic acid precipitation. During trichloroacetic acid precipitation the tube containing the major portion of this sample broke in the centrifuge tube. The recovered material was contaminated with copper salts.

schen and Iwanaga (1966) on trypic digests of fractions of fibrinogen obtained by sulfitolysis show that all of the fractions and, therefore, presumably all of the chains contain carbohydrate. Therefore, six would be the minimum number of glycopeptides if each chain had only one glycopeptide. A value of 6 moles of sialic acid and of 24 moles of hexose per mole of fibrinogen compares well with the values of Mosesson *et al.* (1967) who found 6.1 moles of sialic acid and 27 ± 4.0 moles of hexose per mole of the more soluble human fibrinogen.

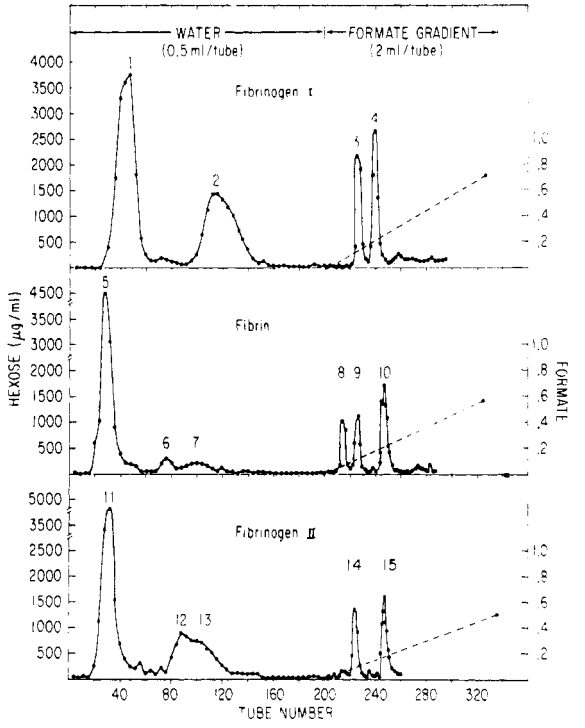

FIGURE 1: Resolution of glycopeptides on Dowex 1 formate. Glycopeptide mixtures (fibrinogen I, 454 mg; fibrinogen II, 350 mg; and fibrin, 353 mg) were applied in 2.5 ml of water, including rinses, to the column of resin bed (0.9 × 70 cm). The column was eluted at 12 ml/hr first with 100 ml of water (0.5 ml/tube) and then with a linear formate gradient at pH 5.0 (2 ml/tube). Fractions were screened by the Loewus (1952) anthrone method.

Regarding the amino acids, the equivalence of lysine and arginine in a given mixture is striking in view of the fact that either lysine or arginine is the carboxy-terminal amino acid of a given glycopeptide according to Haschemeyer *et al.* (1966). Also, glutamic acid is present in the mixtures to the extent of 3 moles/mole of fibrinogen. This fits with the arginine value since glutamic acid occurs only on arginine-containing glycopeptides (Haschemeyer *et al.*, 1966). The data of Mester *et al.* (1965b) also show this equivalence of arginine, glutamic acid, and lysine although it was not commented upon by the authors. These data indicate that all the chains of fibrinogen cannot be pairs with respect to their glycopeptide portion. One of the sets may have an arginyl glycopeptide and one a lysyl glycopeptide.

There appears to be 1 mole less of aspartic acid in the cross-linked fibrin preparation. This indicates that the cross-linking process may have caused the movement of the carbohydrate from one asparagine residue to another position.

Figure 1 shows the result when the glycopeptides were resolved on Dowex 1 formate. Four major glycopeptide fractions were present, the lysyl one containing more than 1 mole of sialic acid (glycopeptides 3, 9, and 14) has not been reported previously (Mester *et al.*, 1967). The finding of four major glycopeptide fractions, not three, is another strong piece of evidence that the duplicate chains are not equivalent in their carbohydrate content.

Sepraphore electrophoresis of the mixtures of glycopeptides at pH 6.4 showed three main bands as Mester *et al.* (1965a) found. However, isolated glycopeptide fractions 2 and 3 (see Figure 1) both have the mobility of the middle band in electrophoresis as do fractions 6, 7, 8, and 9 from fibrin, and 12, 13, and 14 from fibrinogen preparation II. Thus, Dowex 1 afforded further resolution of the mixtures. Glycopeptide fraction 8 from fibrin was not eluted from Dowex 1 with water as were its counterparts from fibrinogen. This greater affinity for Dowex 1 at pH 5 was not reflected in the electrophoretic mobility neither at pH 5 nor at pH 6.4. It moved as the middle band in both instances.

In Table III are shown the actual analyses on the individual glycopeptide fractions in per cent. In most cases the weight of the material can be accounted for by the

TABLE II: Glycopeptide Mixtures and Micromoles[a] per Micromoles of Starting Material.

	Glyco-peptide[b]	Sialic Acid	Hexose	Asp	Glu	Gly	Lys	Arg
Fibrinogen II	5.6	6.1	24	6.0	3.1	3.1	2.4	2.5
Fibrin	5.3	5.9	22	4.9	3.1	1.8	2.4	2.1

[a] All values have been corrected to 100% recovery on the basis of sialic acid by dividing them by 0.7 for fibrinogen and 0.69 for fibrin. [b] A molecular weight of 2500 was used for this calculation.

TABLE III: Isolated Glycopeptides with Analyses in Per Cent.[a]

			Fibrinogen I			
Glycopeptide fraction	1	2			3	4
Yield (mg)	112[b]	64			49	86
Amino acids	15.51	33.86			9.6	18.0
Neuraminic acid	4.9	9.1			21.2	16.2
Hexose	25.7	36.0			37.8	30.0
N-Acetylglucosamine[d]	17.0	27.7			29.8	31.4
Total (%)[a]	63.11	106.66			98.4	95.6
			Fibrin			
Glycopeptide fraction	5	6	7	8	9	10
Yield (mg)	61	7	10	38	48	94
Amino acids	15.34	25.43	14.67	23.87	12.8	19.63
Neuraminic acid	9.84	6.8	8.0	11.4	20.2	16.6
Hexose	45.2	36.0	36.5	40.3	35.7	36.1
N-Acetylglucosamine[d]	34.3	26.4	29.1	31.6	32.6	32.0
Total (%)[a]	104.68	94.63	88.27	107.17	101.3	104.33
Molecular weight (g)				2600	2700	
			Fibrinogen II			
Glycopeptide fraction	11	12	13		14	15
Yield (mg)	80	11	30		64	62
Amino acids	24.30	25.52	20.83		10.74	18.44
Neuraminic acid	9.31	9.31	10.5		17.4	17.1
Hexose	37.7	41.5	40.2		40.3	39.3
N-Acetylglucosamine[d]	33.5	35.7	35.1		35.1	30.4
Total (%)[a]	104.81	112.03	106.63		103.54	105.24
Molecular weight (g)			2300			2500

[a] The theoretical total per cent based on glycopeptide 13 (mol wt 2300) is 109% because of water of hydrolysis. [b] This fraction contained most of the contamination resulting from the mixture of fibrinogen I glycopeptides being broken in the centrifuge. This fraction was treated with Dowex 50-X12 (20–50 mesh) before hydrolysis for amino acids. [c] Anthrone by Loewus (1952) method. The others were done by the phenol–H_2SO_4 method. [d] Calculated from Elson–Morgan hexosamine.

analyses performed. Based on glycopeptide 13 (mol wt 2300) the theoretical total is 109% because it includes water of hydrolysis.

The analytical values are shown as molar ratios in Table IV; aspartic acid was set equal to 1.00 for this calculation.

The presence of cystine in the arginine-containing glycopeptide fractions 2, 6, and 12 must mean these carbohydrate moieties are attached near one of the disulfide bridges of fibrinogen and fibrin. When the glycopeptide fractions were reacted with *C. perfringens* sialidase, both *N*-acetyl and *N*-glycolylsialic acids were released from each glycopeptide. Therefore, those fractions containing 1 mole or less than 1 mole of sialic acid must still be mixtures. The enzyme also removed sialic acid from bovine fibrinogen itself.

When fraction 4 (Figure 1) was reacted first with *C. perfringens* sialidase and then with crude glucosidase

TABLE IV: Isolated Glycopeptides and Molar Ratios of Major[a] Components.

	Fibrinogen I					
	Glycopeptide fraction					
	1	2			3	4
Asp	1.00	1.00			1.00	1.00
Lys	0.79	0.12			0.84	Trace
Arg	0.19	0.61			Trace	0.78
Glu	0.19	0.88			Trace	0.91
Gly	0.29	0.77			Trace	0.81
Val	0.16	0.60			Trace	0.81
Cys[b]	0	0.17			0	0
Neuraminic acid	0.51	0.60			2.1	1.6
Hexose	4.6	4.1			6.4	5.2
N-Acetylglucosamine	2.5	2.6			4.1	4.4
	Fibrin					
	Glycopeptide fraction					
	5	6	7	8	9	10
Asp	1.00	1.00	1.00	1.00	1.00	1.00
Lys	0.91	0.42	0.09	0.16	0.81	0.16
Arg	0.16	0.47	0.85	0.90	0.07	0.79
Glu	0.10	0.94	0.92	0.90	Trace	1.03
Gly	0.10	0.60	0.89	0.80	Trace	0.77
Val	0	0.25	0.17	0.18	0	0.25
Cys[b]	0	0.32	0	0	0	Trace
Neuraminic acid	0.73	0.53	0.93	0.83	1.5	1.5
Hexose	5.8	4.9	7.3	5.0	4.7	5.5
N-Acetylglucosamine	3.6	2.9	4.7	3.2	3.5	3.9
	Fibrinogen II					
	Glycopeptide fraction					
	11	12	13		14	15
Asp	1.00	1.00	1.00		1.00	1.00
Lys	0.71	0.08	Trace		0.78	Trace
Arg	0.20	0.80	0.65		Trace	0.77
Glu	0.08	0.95	0.92		Trace	0.95
Gly	0.34	0.91	0.95		Trace	0.79
Val	0.12	0.05	Trace		Trace	0.06
Cys[b]	Trace	0.20	Trace		Trace	0
Neuraminic acid	0.54	0.65	0.74		1.4	1.4
Hexose	3.8	5.0	4.8		5.4	5.6
N-Acetylglucosamine	2.7	3.5	3.4		3.8	3.5

[a] Thr (as much as 0.38 mole/mole of Asp), Ser, Pro, Ile, Leu, Tyr, Phe, and Ala were also often found. [b] Not corrected for hydrolysis losses; actual value much higher.

(Spiro, 1966) for varying lengths of time, and the products were examined by thin-layer chromatography for both galactose and mannose and by the color reaction for *N*-acetylhexosamine (Reissig *et al.*, 1955), it could be estimated by the quantity of the individual constituents that the sequence from the nonreducing end was sialic acid, galactose, *N*-acetylhexosamine, and mannose in accord with the results Mester (1968) obtained by mild acid hydrolysis. If the sialic acid was not removed, galactose in small amounts and traces of *N*-acetylhexosamine were the only detectable products of the action of crude glucosidase.

Discussion

Mester *et al.* (1963a) isolated three glycopeptides from fibrinogen by electrophoresis of a purified Pronase digest. They were designated A, B, and C on the basis of their electrophoretic mobilities.[1] The two faster ones,

[1] These designations do not correspond to chain designations based on the action of thrombin in which system A chains yield fibrinopeptide A; B chains yield fibrinopeptide B and the T chain is unaffected. As a result, the chain which is unaffected by thrombin appears to contain glycopeptide A (Mester and Moczar, 1965) or IVA (Haschemeyer *et al.*, 1966).

B and C, resembled each other analytically but differed from A which had less sialic acid and more hexose and glucosamine.

Haschemeyer *et al.* (1966) have isolated and characterized three glycopeptides after removal of the sialic acid. They determined the amino acid sequences as: IVA, Asp-Lys; IVB, Gly-Glu-Asp-Arg; and IVC, Glu-Asp-Arg.[1] They have recently separated the chains of fibrinogen and have shown that the T chain yields no arginine-containing glycopeptide but has Asp-Lys as its major component and contains, in addition, a considerable amount of a glycopeptide containing only aspartic acid (Han *et al.*, 1967).

According to L. Mester (personal communication), glycopeptide A has C-terminal lysine and some C-terminal threonine, B and C have C-terminal arginine, and C has N-terminal glycine. Mester and Moczar (1965) also separated the chains by sulfitolysis and were able to characterize two of the three fractions. The *S*-sulfo derivative of the T chain identified by the N-terminal tyrosine was the one with the low sialic acid content (*i.e.*, A with C-terminal lysine).

Gerbeck *et al.* (1967) have raised the question of heterogeneity between the chains of a given pair since the isolated T chains could be further separated into two fractions similar in amino acid and glucosamine contents but different in electrophoretic mobility. The results presented here support the validity of their question since four quite distinct glycopeptide fractions and as many as five or six having only subtle differences from each other have been found (see Figure 1). Another reason to speculate that the duplicate chains are not identical is the equivalence of arginine and lysine in the glycopeptide mixtures. According to Haschemeyer *et al.* (1966), either lysine or arginine is the residue next to the carbohydrate carrying asparagine. The data presented here (Figure 1, Table IV) also show a given glycopeptide fraction having either lysine or arginine with only small amounts of the other present. The simplest interpretation of these data is that there are two basic types of glycopeptides depending upon whether the asparagine carrying the carbohydrate is next to a lysyl or an arginyl residue in the peptide chain. To occur in pairs there must then be two with lysine and four with arginine or *vice versa*, but not three and three. In this light, the equivalence of arginine and glutamic acid with lysine indicates the two individual chains, at least in one of the pairs, are quite different, one containing lysine next to the β-amide-linked asparagine and the other containing arginine.

Glycopeptide fractions 2, 6, and 12 (Figure 1) may be located very near to a disulfide bond since they contain cystine.

The greater affinity of glycopeptide fraction 8 for the Dowex 1 resin compared with glycopeptide fractions 2 and 12–13, which are presumed to be its fibrinogen counterparts, is unexplained. Nevertheless, the occurrence of this glycopeptide is a further indication that the cross-linking process affected a carbohydrate chain without changing the total carbohydrate content of fibrinogen.

Acknowledgments

We are indebted to Dr. J. E. Folk for the transglutaminase and to Drs. Filadelfo Irreverre, A. J. Osbahr, E. Gross, and John Morrell for performing amino acid analyses on the isolated fractions. Miss Hallie Cross of Western Maryland College performed the experiments involving the action of sialidase and β-glucosidase on the glycopeptides during her summer employment in this laboratory. Dr. W. R. Carroll and Mr. E. R. Mitchell carried out the ultracentrifugal experiments and the calculation of molecular weights.

References

Bagdy, D., and Szara, I. (1955), *Acta Physiol. Acad. Sci. Hung. 7*, 179.

Blombäck, B. (1958), *Arkiv Kemi 12*, 99.

Brown, M. (1963), *Vox Sanguinis 8*, 104.

Cassidy, J. T., Jourdian, G. W., and Roseman, S. (1966), *Methods 8*, 680.

Chandrasekhar, N., and Laki, K. (1964), *Biochim. Biophys. Acta 93*, 392.

Chandrasekhar, N., Osbahr, A. J., and Laki, K. (1964), *Biochem. Biophys. Res. Commun. 15*, 182.

Chandrasekhar, N., Osbahr, A. J., and Laki, K. (1966), *Biochem. Biophys. Res. Commun. 23*, 757.

Chandrasekhar, N., Warren, L., Osbahr, A. J., and Laki, K. (1962), *Biochim. Biophys. Acta 63*, 337.

Cynkin, M. A., and Haschemeyer, R. H. (1964), *Federation Proc. 23*, 273.

Dreywood, R. (1946), *Ind. Eng. Chem. Anal. Ed. 18*, 499.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Elson, L. A., and Morgan, W. T. J. (1933), *Biochem. J. 27*, 1824.

Fischer, F. G., and Dörfel, H. (1955), *Z. Physiol. Chem. 301*, 224.

Folk, J. E., and Cole, P. W. (1966), *J. Biol. Chem. 241*, 5518.

Gerbeck, C. M., Yoshikawa, T., and Montgomery, R. (1967), *Federation Proc. 26*, 537.

Han, L.-C., Trindle, M., Haschemeyer, R. H., and Cynkin, M. A. (1967), *Federation Proc. 26*, 607.

Haschemeyer, R. H., Cynkin, M. A., Han, L.-C., and Trindle, M. (1966), *Biochemistry 5*, 3443.

Henschen, A., and Iwanaga, S. (1966), *XI Intern. Hematolog. Congr., Sydney*.

Hörmann, H., and Gollwitzer, R. (1964), *Proc. Colloq. Protides Biol. Fluids 11*, 332.

Laki, K. (1951a), *in* Blood Clotting and Allied Problems, Flynn, J. E., Ed., New York, N. Y., Josiah Macy, Jr. Foundation, p 217.

Laki, K. (1951b), *Arch. Biochem. 32*, 317.

Laki, K. (1968), *in* Fibrinogen, Laki, K., Ed., New York, N. Y., Marcel Dekker, p 1.

Laki, K., and Chandrasekhar, N. (1963), *Nature 197*, 1267.

Laki, K., and Mester, L. (1962), *Biochim. Biophys. Acta 57*, 152.

Loewus, F. A. (1952), *Anal. Chem. 24*, 219.

Mester, L. (1968), *in* Fibrinogen, Laki, K., Ed., New York, N. Y., Marcel Dekker, p 165.
Mester, L., and Moczar, E. (1965), *Compt. Rend. 260*, 2617.
Mester, L., Moczar, E., and Laki, K. (1963b), *Compt. Rend. 256*, 307.
Mester, L., Moczar, E., Medgyesi, G., and Laki, K. (1963a), *Compt. Rend. 256*, 3210.
Mester, L., Moczar, E., and Szabados, L. (1967), *Compt. Rend. 265*, 877.
Mester, L., Moczar, E., Vass, G., and Szabados, L. (1965a), *Compt. Rend. 260*, 2342.
Mester, L., Moczar, E., Vass, G., and Szabados, L. (1965b), *Pathol. Biol. Somaine Hop. 13*, 540.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Mosesson, M. W., Alkjaersig, N., Sweet, B., and Sherry, S. (1967), *Biochemistry 6*, 3279.
Partridge, S. M. (1949), *Nature 164*, 443.
Raisys, V., Molnar, J., and Winzler, R. J. (1966), *Arch. Biochem. Biophys. 113*, 457.
Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.
Rosenberg, A., and Carman, R. H. (1964), *Nature 204*, 994.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Spiro, R. G. (1962), *J. Biol. Chem. 237*, 382.
Spiro, R. G. (1966), *Methods Enzymol. 8*, 26.
Swann, D. A., and Balazs, E. A. (1966), *Biochim. Biophys. Acta 130*, 112.
Szara, S., and Bagdy, D. (1953), *Biochim. Biophys. Acta 11*, 313.
Tyler, H. M. (1966), *Nature 210*, 1045.
Tyler, H. M., and Laki, K. (1966), *Biochem. Biophys. Res. Commun. 24*, 506.
Warren, L. (1959), *J. Biol. Chem. 234*, 1971.
Warren, L. (1960), *Nature 186*, 237.

Molecular Conformation of Chymotrypsinogen and Chymotrypsin by Low-Angle X-Ray Diffraction*

W. R Krigbaum and R. W. Godwin†

ABSTRACT: Low-angle diffraction measurements are reported for chymotrypsinogen A and four chymotrypsins in 0.1 M NaCl at pH 7.0. Chymotrypsinogen is found to have a radius of gyration, R, of 18.1 Å and an axial ratio 2.0. The equivalent scattering ellipsoid has semiaxes 33.0 and 16.5 A, and a surface-to-volume ratio of 0.155 A^{-1}. The latter quantity agrees with that measured for the molecule in solution, 0.160 $Å^{-1}$, indicating that chymotrypsinogen has a smooth surface free of major protuberances. The principal product of slow activation, α-chymotrypsin, has very nearly the same size and shape (R = 18.0 Å and axial ratio 2.0), while the rapid activation end product, δ-chymotrypsin, is slightly larger (R = 19.0 Å) and somewhat more symmetrical (axial ratio 1.8). These results indicate that the activation of chymotrypsinogen is not accompanied by a gross change of the molecular conformation in solution. Two minor products of the slow activation process, β- and γ-chymotrypsin, are aggregated to some extent in 0.1 M NaCl. Chymotrypsinogen and chymotrypsin also have nearly the same molecular conformations in the crystalline state, but these are significantly smaller and more symmetrical than those deduced from our solution studies.

Proteolytic enzymes are secreted in the form of an inactive precursor, or zymogen. Zymogen activation, which creates the active enzyme, involves splitting of the polypeptide chain at specific sites. Neurath, in an excellent review of proteolytic enzymes (Neurath, 1964), has pointed out the importance of obtaining information concerning the conformational changes which accompany zymogen activation if one is to understand how the specificity site is created in this process.

One such zymogen is chymotrypsinogen, an inactive pancreatic enzyme first isolated by Kunitz and Northrop (1933). These workers also discovered that chymotrypsinogen is converted into the active pancreatic enzyme, chymotrypsin, by minute amounts of trypsin. One obtains from cattle pancreatic juice approximately equal quantities of two chymotrypsinogens designated A and B. At pH 8 the former is cationic, while the latter is anionic. Structural studies have been largely confined to chymotrypsinogen A, and this form is implied in all further references to chymotrypsinogen.

Both trypsin and chymotrypsin are capable of hydrolyzing the peptide bonds of chymotrypsinogen, so that different conditions of hydrolysis lead to different active forms. Slow activation of chymotrypsinogen in the

* From the Department of Chemistry, Duke University, Durham, North Carolina. *Received April 4, 1968.*
† National Aeronautics and Space Administration fellow.