LAMPREY FIBRINOPEPTIDE B IS A GLYCOPEPTIDE

R. F. Doolittle and B. A. Cottrell

Department of Chemistry, University of California, San Diego

La Jolla, California 92037

Received August 23,1974

SUMMARY: One of the peptides released from lamprey fibrinogen during its transformation into fibrin has been found to contain covalently bound carbohydrate. The peptide, which also contains tyrosine O-sulfate, corresponds to the mammalian fibrinopeptide B and is the amino-terminal segment of the lamprey fibrinogen β-chain. As noted previously, this peptide is the only one released when lamprey fibrinogen is clotted by mammalian thrombin. Of the more than fifty sets of fibrinopeptides characterized from various species, this is the first one found to contain carbohydrate.

The lamprey--sometimes called the lamprey eel--is the most primitive vertebrate extant. As such, there is considerable interest in its proteins, and especially those which seem unique to vertebrates. Previous studies (1-4) have shown that this creature has a fibrinogen which is homologous to the mammalian type in that it has three pairs of non-identical chains, two pairs of which have fibrinopeptides as their amino-terminal segments. Only one pair of these--previously designated the fibrinopeptide B--is released during clotting by mammalian thrombins (3,4). This is a very large peptide (37 amino acid residues) and contains the unusual amino acid tyrosine O-sulfate. We now report that this fibrinopeptide also contains covalently bound carbohydrate.

EXPERIMENTAL

Lampreys (Petromyzon marinus) were collected in various New England rivers and streams during the 1972, 1973 and 1974 spring spawning runs. Blood collection and processing were carried out as described previously (3). Fibrinogen was prepared by a cold ethanol precipitation scheme (5). It was greater than 95% clottable with either lamprey (3) or bovine (Parke-Davis)

thrombin and gave only three bands (corresponding to the α-, β-, and γ-chains) upon SDS-gel electrophoresis under reducing conditions (6,7). Comparison of the subunit structure of lamprey fibrinogen and fibrin with mammalian types revealed that lamprey fibrinogen has very large α-chains, as reported elsewhere (8-10). The β-chains were also apparently larger than their mammalian counterparts, although the size difference disappeared dramatically after clotting with either lamprey or mammalian thrombin (Fig. 1). Carbohydrate-staining by the periodic acid-Schiff reagent (11) showed that only the β- and γ-chains were positive by this method, just as is the case in mammalian fibrinogens (12,13). Moreover, the lamprey fibrinogen β-chain staining was particularly strong, although the corresponding intensity in fibrin was markedly less (Fig. 1). Semi-quantitation of this diminution was attained by photographing the PAS-stained gels and scanning the negatives on a Joyce-Loebl microdensitometer. The scans were traced, cut out and weighed. In the lamprey case, the β/γ ratio for PAS-positive material shifted from 2.5 to 1.5 upon conversion to fibrin with either thrombin. The human and bovine ratios (1.8 and 1.3, respectively) did not change significantly as a result of clotting.

The lamprey fibrinopeptide B was isolated by clotting relatively large batches of purified fibrinogen (200-400 mg) with bovine thrombin, winding out the fibrin, and passing the clot liquor over a Sephadex G-50 column (2.5 x 100 cms) equilibrated with 0.1 M ammonium bicarbonate in order to separate the peptide material from the small amounts of unclotted protein. The peptide pool was concentrated by freeze-drying and examined by paper electrophoresis at pH 4.1 (300 V, 4 hrs). A single anodic band appeared which was both ninhydrin-positive and arginine-positive (14). The amino acid composition of the eluted material was in substantial agreement with that reported previously (4), except that it was observed that the material also contained glucosamine. Accordingly, a quantitative determination of neutral sugars was undertaken by the phenol-sulfuric acid

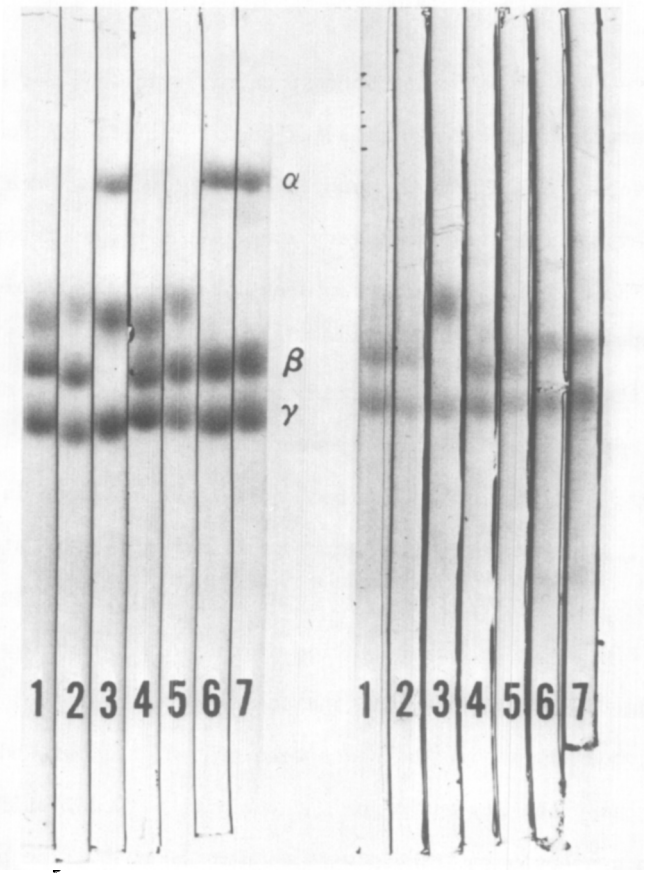

Fig. 1. SDS-gels of bovine, human and lamprey reduced fibrinogens and fibrins. Left hand set stained with Comassie blue for protein, right hand set stained with PAS for carbohydrate. 1, bovine fibrinogen; 2, human fibrinogen; 3, lamprey fibrinogen; 4, bovine fibrin; 5, human fibrin; 6, lamprey fibrin formed with bovine thrombin; 7, lamprey fibrin formed with lamprey thrombin. Only the α-, β- and γ-chains of lamprey fibrin are denoted with Greek letters.

procedure (15) and orcinol method (16), as well as a measurement of sialic acid (17). For comparison purposes, preparations of sheep fibrinopeptide B and human fragment E (derived from a plasmin digest of fibrinogen) were also analyzed. The former was negative for all carbohydrate tests; the latter, the carbohydrate composition of which has previously been reported in a different vein (18), gave approximately the expected values. Qualitative identification of the constituent neutral sugars in the lamprey fibrinopeptide B was obtained after mild acid hydrolysis (4N HCl, 2.5 hrs, 108°) followed by passage over a small (6.0 ml) Dowex 50-X4 column (H+).

The concentrated eluates were chromatographed in an ethyl acetate-pyridine-water (48:20:16) system and the reducing sugars present exposed by a silver nitrate stain (19). D-glucose, D-galactose, D-mannose and L-fucose were run as reference materials.

The lamprey fibrinopeptide B is composed of slightly more than one-third carbohydrate (Table I). Its molecular weight is approximately 6300, 3950 of which is contributed by its 37 amino acids. Neutral sugars amount to 7 residues per mole, based on a sucrose (2 residue) standard. Sialic acids were found to be present at approximately 2 residues per mole, the same value as was determined for human fragment E (mol. wt = 2 x 25,000). Glucosamine, as measured on the short column of a standard amino acid analyzer after a hydrolysis time of 15 hours, was found to be present at 2-3 residues per mole in the case of both lamprey fibrinopeptide B and human fragment E. Thus, the total carbohydrate in the lamprey fibrinopeptide B consists of a cluster of a dozen sugar units, the total molecular weight contribution of which ought to be about 2350. The neutral sugars detected by paper chromatography were galactose, mannose and fu-

TABLE I

Chemical Composition of Lamprey Fibrinopeptide B

	Residues/Mole	Molecular Wt. Contribution	Percent Mass
Amino acids	37	3950	(63)
Carbohydrate			
Neutral sugars[a]	7 (7.2)		
Sialic acid	2 (1.9)		
Glucosamine	3 (2.6)		
Total sugars	12	2350[b]	(37)
Total glycopeptide		6300	(100)

[a] Identified as galactose, mannose and fucose.

[b] Glucosamine presumed to be in N-acetyl form (20).

cose, the latter being present in relatively small amounts. Finally, the presence of glucosamine--but not galactosamine--in the lamprey fibrinopeptide B suggests that the carbohydrate moeity is attached to an asparagine residue (20).

DISCUSSION

The lamprey fibrinopeptide B had been studied previously, including that half of its amino acid sequence at its amino terminus (4). At the time, the relatively small amounts of material available were utilized in a manner consistent with the facilities available, the bulk of the material being devoted to stepwise degradation from the amino-terminal. The carbohydrate was missed, even though a cursory staining test for sugar was employed. In retrospect, the method and amounts used were both inadequate to the task (4). Still, it was a shock a decade later to stumble on to this material, since no previous fibrinopeptide, A or B, had ever been found to have carbohydrate, even though upwards of 50 species have had their fibrinopeptides characterized.

The presence of carbohydrate in the lamprey fibrinopeptide B explains at least one puzzling aspect, while posing some new ones. For example, the large apparent size difference between the β-chains of lamprey fibrinogen and fibrin is now understandable, for, not only does the carbohydrate make the fibrinopeptide half again as big, but its presence on the virgin β-chain must also contribute to the "carbohydrate drag" frequently observed on SDS-gels (21).

The physiological significance of this glycopeptide is still unclear. In this regard, it is not yet possible to determine if the release of this carbohydrate is absolutely essential for the transformation of lamprey fibrinogen into fibrin, since lamprey thrombin releases the fibrinopeptides A and B at approximately the same rate (3). On another tack, attempts to clot lamprey fibrinogen by treatment with sialidase (Calbiochem) have thus far proved futile.

Finally, any hope of finding a simple structural consequence for fibrinopeptide removal must take account of the fact that the lamprey fibrinopeptides A and B represent the smallest (A = 6 residues) and largest (B = 6300 mol. wt.) fibrinopeptides yet found. Furthermore, the fibrinopeptide A, which has the simple sequence Asp-Asp-Ile-Ser-Leu-Arg, does not contain carbohydrate (22). It is clearly a fibrinopeptide A, since the α-chain of which it comprises the amino-terminus is strongly homologous to mammalian α-chains (22). Some other extraordinary features of this molecule--including some preserved through 400 million years of natural selection and others totally unrecognizable in mammalian systems--will be discussed in a separate publication (22).

ACKNOWLEDGMENTS

We would like to thank Marcia Wilson for assistance in preparing lamprey fibrinogen and Ken Cassman for isolating human fragment E. This work was supported by N.I.H. Grants No. HE 12,759 and GM 17,702.

REFERENCES

1. Doolittle, R.F., Oncley, J.L. and Surgenor, D.M., J. Biol. Chem., 237:3123 (1962).
2. Doolittle, R.F., Lorand, L. and Jacobsen, A., Biochim. Biophys. Acta, 69:161 (1963).
3. Doolittle, R.F., Biochem. J., 94:735 (1965)
4. Doolittle, R.F., Biochem. J., 94:742 (1965).
5. Doolittle, R.F., Schubert, D. and Schwartz, S.A., Arch. Biochem. Biophys., 18:456 (1967).
6. Weber, K. and Osborn, M., J. Biol. Chem., 244:4406 (1969).
7. McKee, P.A., Mattock, P. and Hill, R.L., Proc. Natl. Acad. Sci., U.S., 66:738 (1970).
8. Doolittle, R.F., Adv. Protein Chem., 27:1 (1973).
9. Murtaugh, P.A., Halver, J.E. and Gladner, J.A., Fed. Proc., 33:1474abs (1974).
10. Doolittle, R.F. and Wooding, G.L., Biochim. Biophys. Acta, In Press.
11. Zacharius, R.M., Zell, T.E., Morrison, J.H. and Woodlock, J.J., Anal. Biochem., 30:148 (1969).
12. Gaffney, P.J., Biochim. Biophys. Acta, 263:453 (1972).
13. Pizzo, S.V., Schwartz, M.L., Hill, R.L. and McKee, P.A., J. Biol. Chem., 247:636 (1972).
14. Yamada, S. and Itano, H.A., Biochim. Biophys. Acta, 130:538 (1966).

15. Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., Anal. Chem., 28:350 (1956).
16. Hewett, L.F., Biochem. J., 31:360 (1937).
17. Warren, L., J. Biol. Chem., 234:1971 (1959).
18. Blombäck, B., Gröndahl, N.J., Hessel, B., Iwanaga, S. and Wallén, P., J. Biol. Chem., 248:5806 (1973).
19. Smith, I., "Chromatographic and Electrophoretic Techniques" Vol 1. Interscience, New York, 1960.
20. Spiro, R.G., Adv. Protein Chem., 27:349 (1973).
21. Bretscher, M.S., Nature New Biology, 231:229 (1971).
22. Doolittle, R.F. and Cottrell, B.A. In Preparation.