AMINO TERMINAL AMINO ACID SEQUENCES AND CARBOHYDRATE OF THE TWO MAJOR FORMS OF RABBIT PLASMINOGEN

F. J. Castellino+, G. E. Siefring, Jr., J. M. Sodetz and R. K. Bretthauer
Department of Chemistry, Program in Biochemistry and Biophysics, University of Notre Dame, Notre Dame, Indiana 46556

Received June 20,1973

SUMMARY: Two major forms of plasminogen exist in the plasma of many animal species and are distinguished by their affinities for certain antifibrinolytic amino acids. Quantitative end group analysis demonstrated that each isolated form of rabbit plasminogen possessed a single amino terminal residue of glutamic acid. Amino acid sequence analysis indicated that at least the first twelve amino terminal amino acids were identical in the two forms. The unique amino terminal sequence obtained for each form was NH_2-glu-pro-leu-asp-asp-tyr-val-asn-thr-gln-gly-ala-. Analysis of the carbohydrate content of each major plasminogen form revealed some striking differences. The first major form of rabbit plasminogen isolated from affinity chromatography columns contained 1.5-1.7 percent neutral carbohydrate and 3.0-3.3 moles of sialic acid per mole of protein. The second major form of rabbit plasminogen isolated from affinity chromatography columns contained 0.6-0.8 percent neutral carbohydrate and 1.8-2.2 moles of sialic acid per mole of protein.

Recent studies from our laboratory (1) have shown that two major forms of rabbit plasminogen can be isolated from rabbit plasma by a modification (2) of the Deutsch and Mertz affinity chromatography technique (3). These forms appear to be very similar in their molecular weights (1), amino acid compositions (1) and their activation properties by streptokinase and urokinase. On the other hand, the two rabbit plasminogen forms are distinctly different in their charge properties. The first major plasminogen form eluted

+ These studies were supported by Grant HL-13423 from the N.I.H. and a grant-in-aid from the Indiana and American Heart Associations.

from affinity chromatography columns possesses 5 isozymes with an isoelectric pH range of 6.20-7.78. The second major form eluted from affinity chromatography columns also possesses 5 isozymes but the isoelectric pH range is 6.95-8.74 (1).

Several years ago, Wallen (4) demonstrated the existence of two major forms of plasminogen in the plasmas of several species. Wallen and Wiman (5) have isolated two main forms of human plasminogen by DEAE-Sephadex chromatography and concluded that one form was an amino terminal proteolytic product of the other. The parent form possessed an amino terminal glutamic acid residue whereas the other form possessed lysine and valine amino terminal residues. This was subsequently verified by Rickli and Otavsky (6), who also demonstrated that only the glutamic acid form of human plasminogen is isolated by affinity chromatography.

It was apparent from our earlier studies (1) that the two forms of rabbit plasminogen were not artifacts produced by proteolysis, although this was not conclusively demonstrated. We therefore wished to examine this point by amino acid sequence analysis as well as to determine whether the carbohydrate moiety played any role in differentiating the two major forms of rabbit plasminogen.

Materials and Methods - The two forms of rabbit plasminogen were purified by affinity chromatography of fresh citrated plasma as previously described (1).

Quantitative determinations of the amino terminal amino acids in rabbit plasminogen were performed as described by Stark (7) on a Beckman 117 Amino Acid Analyzer. Protein concentrations were determined at the cyclization step by

Table I

Amino Terminal End Group Analysis of Rabbit Plasminogen

Protein	Amino Acid	Moles of Amino Acid Per Mole of Protein
Plasminogen F-1[a]	glutamic acid	0.95
	arginine[b]	0.08
Plasminogen F-2[a]	glutamic acid	1.04
	arginine[b]	0.07

[a]Refers to fraction 1 and fraction 2 plasminogen isolated from affinity chromatography (1).

[b]Highest impurity.

amino acid analysis of a hydrolysate of an aliquot from the cyclization step.

Automatic Edman degradations of rabbit plasminogen fractions 1 and 2 were performed with a Beckman Model 890 B Sequencer. The program was structured to the basic Quadrol modifications recommended by Hermodson (8). The amino acid phenylthiohydantoin derivatives were identified with the aid of a Beckman GC 45 gas chromatograph on a 2 mm x 4 ft glass column packed with SP-400 (10% on Supelcoport, 100/120 mesh, Supelco, Inc.). Silica thin layer chromatography, employing solvent XM (9), was used for discriminations between threonine and glycine and between threonine and proline derivatives.

Sialic acid was determined by the thiobarbituric acid assay (10) after releasing the sialic acid from the protein with 0.1 N H_2SO_4 at 80° for one hour. The total neutral

Table II

Automated Edman Degradation of Rabbit Plasminogen Fractions 1 and 2

Residue Number	Fraction 1[a] amino acid	n moles	Fraction 2[a] amino acid	n moles
1	glutamic acid	34	glutamic acid	38
2	proline	33	proline	36
3	leucine	26	leucine	34
4	aspartic acid	17	aspartic acid	23
5	aspartic acid	28	aspartic acid	32
6	tyrosine	26	tyrosine	29
7	valine	30	valine	23
8	asparagine[b]	--	asparagine[b]	--
9	threonine[b,c]	--	threonine[b,c]	--
10	glutamine[b]	--	glutamine[b]	--
11	glycine[c]	36	glycine[c]	29
12	alanine	34	alanine	31

[a] 100 nmoles uncorrected for residual salt and moisture taken for analysis.

[b] These amino acids were identified qualitatively.

[c] These assignments were confirmed by thin layer chromatography.

sugar content of each plasminogen form was determined by the phenol-sulfuric acid method (11).

Results - Table I lists the quantitative recoveries of the amino terminal amino acid residues from each rabbit plasminogen form. The results are entirely unambiguous in that glutamic acid was found as the sole amino terminal amino acid of each plasminogen form.

Table II gives the sequential amino acid release from the amino terminus of each rabbit plasminogen fraction. The absolute yields of the amino acids are low but constant. It is noted that the recovery of asp_5 is greater than asp_4. This is likely due to incomplete reaction in the coupling or cleavage step. The residual NH_2-terminal asp_4 unreacted in step 4 would be added to asp_5 in the fifth cycle. The lower than desired absolute yields cannot be explained. However, this is not uncommonly observed. Even lower yields were reported in analysis of human plasminogen (12). In our case part of the low yields can be explained by the moisture content of the sample, which was not corrected. Partial blocking of the NH_2-terminal amino acid in plasminogen is ruled out based on the quantitative recovery of amino terminal glutamic acid by Stark's method.

Table III gives the average sialic acid and neutral carbohydrate content of each rabbit plasminogen form. The sialic acid ranges represent an analysis of 10 separate determinations on different preparations of each form. The neutral carbohydrate content is based upon analysis of 3 different preparations of each rabbit plasminogen form.

Discussion - The results presented in this manuscript clearly show that the two affinity chromatography isolated forms of rabbit plasminogen do not differ in their amino terminal amino acid sequence. Thus one form cannot arise from amino terminal proteolysis of the other. Further, since amino acid sequence analysis on each plasminogen form indicated only a single amino acid at each step of the sequence, these results also demonstrate that the 5 isozymes within

Table III

Carbohydrate Analysis of Rabbit Plasminogen

Protein	Sialic Acid Content moles/mole	Neutral Sugar Content % (w/w)
Plasminogen F-1	3.0 - 3.3	1.5 - 1.7
Plasminogen F-2	1.8 - 2.2	0.6 - 0.8

each affinity chromatography form must possess the same amino terminal sequence. The question arises at this point whether the two plasminogen forms could be artifacts due to hydrolysis at the carboxyl terminus or through modification of the carbohydrate moiety. Both of these possibilities can be ruled out by our whole animal studies, soon to be submitted for publication, which conclusively show that both forms of plasminogen must exist as such in the plasma, are not artifacts of the purification, and are not interconverted. Therefore, the two forms of human plasminogen obtained by Wallen and Wiman (5) should not be confused with the two forms of plasminogen obtained in this study. Clearly, they have isolated proteolytically digested plasminogen as one form. This is not the case here.

Carbohydrate analysis of the two forms show interesting differences. Plasminogen fraction 1, which possesses the lower isoelectric forms of plasminogen, contains one additional residue of sialic acid over fraction 2 plasminogen, which contains the higher isoelectric forms. All the isozymes within each plasminogen form contain the same number of

sialic acid residues as the parent form. The sialic acid content therefore may represent a basis for the charge differences in the two major plasminogen forms but is not a basis for the charges differences in the isozymes within each form. Carbohydrate differences in the two major plasminogen forms are also evident upon analysis of the neutral carbohydrate. There is twice as much neutral carbohydrate in rabbit plasminogen fraction 1 as in plasminogen fraction 2. Therefore, we feel that the occurrence of the two major forms of rabbit plasminogen may be due in part to differences in the carbohydrate moiety. This does not appear to be the case for the individual isozymes within each form.

Acknowledgements - We wish to thank Dr. Howard Steinman for performing the automatic amino acid sequence analyses.

REFERENCES

1. Sodetz, J. M., Brockway, W. J. and Castellino, F. J., Biochemistry 11, 4451 (1972)
2. Brockway, W. J. and Castellino, F. J., Arch. Biochem. Biophys. 151, 194 (1972).
3. Deutsch, D. and Mertz, E. T., Science 170, 1095 (1970).
4. Wallen, P., Ark. Kemi. 19, 69 (1962).
5. Wallen, P. and Wiman, B., Biochim. Biophys. Acta 257, 122 (1972).
6. Rickli, E. E. and Otavsky, W. I., Biochim. Biophys. Acta 295, 301 (1973).
7. Stark, G. R., Methods in Enzymology 11, 125 (1967).
8. Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H. and Walsh, K. A., Biochemistry 11, 4493 (1972).
9. Inagami, T. and Murakami, K., Anal. Biochem. 47, 501 (1972).
10. Warren, L., J. Biol. Chem. 234, 1971 (1959).
11. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., Anal. Chem. 28, 350 (1956).
12. Robbins, K. C., Bernabe, P., Arzadon, L., and Summaria, L., J. Biol. Chem. 247, 6757 (1972).