The Role of Sialic Acid in the Determination of Distinct Properties of the Isozymes of Rabbit Plasminogen*

(Received for publication, June 7, 1974)

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SUMMARY

Previous studies from our laboratory have resulted in the separation of two major forms of plasminogen from rabbit plasma. These two forms differ in their affinity characteristics for antifibrinolytic amino acids, metabolic survival times in the circulation, charge characteristics of the subforms resolved from each major form, and sialic acid content. In order to investigate the role of sialic acid in determining these distinct properties of the plasminogen forms, we have studied these same properties of the asialo rabbit plasminogen forms. We find that the charge differences between the two forms are essentially abolished upon removal of the sialic acid. The number of subforms resolved from each major form is decreased as an effect of removal of the sialic acid, but the remaining subforms possess greatly increased isoelectric points. Contrarily, the binding of each plasminogen to antifibrinolytic amino acids appears no different from normal. The metabolic survival times of the asialoplasminogen forms are not greatly different from each other but are significantly lower than normal, a phenomena also found for many other circulating proteins. These studies suggest that the charge differences between the two forms of rabbit plasminogen are incidental to their functional differences.

We have previously shown that two major forms of rabbit plasminogen can be purified from rabbit plasma by affinity chromatography (1). Each major form can be resolved into five subforms which possess distinct isoelectric points with some staggered overlapping (2). Although the molecular weights (2), amino acid compositions (2), and NH₂-terminal amino acid sequences (3) of the two forms are similar, other properties such as the sialic acid content (3), binding to antifibrinolytic amino acids, and circulatory survival times (4) show interesting differences. Many of the distinct properties of the two major plasminogen forms could be related in principle to the sialic acid differences. For example, the differential charge characteristics of the two forms could be a direct result of the sialic acid differences especially since the form with the greater content of sialic acid possesses an acid shift in the isoelectric points of the subforms. With regard to the subforms, it is possible that some of these may also result from sialic acid differences. It is also interesting that the form with the greater content of sialic acid also survives in the circulation longer than the other form. Ashwell and colleagues (5, 6) have proposed a role for sialic acid in protection of the protein from uptake and consequent degradation by the liver.

Due to the potentially interesting relationships of the sialic acid differences to the observed differences in properties of the two major rabbit plasminogen forms we decided to investigate whether this relationship did in fact exist. Our approach to the problem was to determine whether the differential properties of each form of rabbit plasminogen were conserved upon removal of the sialic acid. This manuscript is a result of these studies.

EXPERIMENTAL PROCEDURES

Proteins-Each major form of rabbit plasminogen as well as the subforms studied were purified as described in an earlier report from this laboratory (2).

Neuraminidase (Vibrio cholerae) was purchased from General Biochemicals in vials containing 500 units per ml. These were stored as directed and used without further purification.

Lactoperoxidase was purchased from Calbiochem and coupled to Sepharose 4B as described by David (7).

Asialo rabbit plasminogen Forms 1 and 2 were prepared by dissolving the required plasminogen to 5 mg per ml in a buffer consisting of 0.1 M acetic acid, 0.1 M lysine, 0.002 M CaCl₂, pH 5.6. To 2.5 ml of this solution, 2.5 ml of the stock neuraminidase were added, and the solution was allowed to incubate for 9 hours at 37°. At this time diisopropyl fluorophosphate was added such that the final concentration was 0.01 M. The solution was incubated at 0° for 2 hours. This solution was dialyzed in the cold room against 0.1 M phosphate, pH 8, for 2 days. The asialoplasminogen was then purified by small affinity chromatography columns in an equivalent manner to the original purification of this material. When these conditions are rigorously followed, no proteolytic cleavage will occur in plasminogen. This problem was studied by sodium dodecyl sulfate-β-mercaptoethanol gel analysis.

Static Acid Content of Subforms of Rabbit Plasminogen Forms 1 and 2—This was determined by dissolving the desired subform at 2 mg per ml in 0.1 N H₂SO₄. The samples were then hydrolyzed in sealed tubes at 80° for 1 hour. A 0.5-ml aliquot of the hydrolysate was quantitated for sialic acid by the thiobarbituric assay of Warren (8).

Affinity Chromatography Profiles of Normal and Asialoplasminogen

* This work was supported by Grants HL-13423 and HL-15747 from the National Heart and Lung Institute, National Institutes of Health and a cooperative grant-in-aid from the Indiana and American Heart Associations.

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gens—These were obtained by preparing mixtures of the two forms. Approximately 0.6 mg of normal rabbit plasminogen Form 1 and 1.2 mg of normal rabbit plasminogen Form 2 were dissolved in 2 ml of a solution consisting of 0.1 M phosphate, 0.15 M NaCl, pH 8.0. This mixture was passed over a column (1 X 11 cm) of Sepharose 4B. 1-lysine, prepared as previously described (2). The column was then washed with 25 ml of 0.3 M phosphate, pH 8.0, and the plasminogen forms were eluted with a linear gradient of \( \epsilon \)-aminocaproic acid, exactly as described previously (4). The same procedure was used to obtain affinity chromatography profiles of the asialo rabbit plasminogen forms.

Suission of Plasminogen—This was accomplished with \( ^{125} \text{I} \) by the solid state lactoperoxidase procedure (7). Our lactoperoxidase carrier ampholytes. Our procedures have been described earlier (2). Isoelectric points were obtained by refocusing the peaks on narrow pH gradients.

Sedimentation Velocity Analysis of Each Native and Asialo Form of Rabbit Plasminogen—This was performed at 20°C on a Beckman model E analytical ultracentrifuge equipped with scanner optics. Our procedures for this have been detailed earlier (1).

Sodium Dodecyl Sulfate-Mercaptoethanol Polyacrylamide Gel Electrophoresis—This was performed as described by Weber and Osborn (9).

RESULTS

Fig. 1 shows sodium dodecyl sulfate-\( \beta \)-mercaptoethanol gels for each native and iodinated native affinity chromatography form of rabbit plasminogen as well as gels for each asialo and iodinated asialo form of these proteins. Clearly, treatment with neuraminidase as well as the enzyme and reagents used for iodination did not result in any proteolytic cleavages in plasminogen under our conditions. Table I shows the rate of loss of sialic acid from each rabbit plasminogen form upon treatment with neuraminidase as described under "Methods." Loss of sialic acid was monitored by the thiobarbituric assay (8) on aliquots of the reaction. The content of sialic acid of plasminogen, as determined in this table, is slightly higher by almost 0.3 to 0.4 mole per mole than we reported earlier (3). However, we feel this variation exists from preparation to preparation and the fact that the two plasminogen major forms differ in sialic acid content is not altered. At the conclusion of the neuraminidase reaction, each plasminogen was purified by affinity chromatography, and an aliquot was hydrolyzed by sulfuric acid to release any residual sialic acid. Assays at this point demonstrated that essentially no sialic acid remained on the proteins. Incubation of each asialo plasminogen with urokinase demonstrated that these plasminogen derivatives could be activated to plasmin.

Fig. 2 shows some pH 4.3 gels on mixtures of each native and asialo rabbit plasminogen major form. It appears clear that the charge differences which exist in the two native forms are greatly reduced and practically abolished upon removal of the sialic acid. This finding is corroborated by the isoelectric focusing profiles shown for each asialo major form of rabbit plasminogen in Fig. 3. In this figure, two to three subforms are resolved from each major form of the desialylated rabbit plasminogen. The isoelectric points of each subform, which were obtained by refocusing each peak on narrow pH gradients, are summarized in Table II. It appears clear that when the two forms are com-

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Table I

| Time of treatment with neuraminidasea | Sialic acid liberated |
|--------------------------------------|----------------------|
| | F-1b | F-2c |
| hrs | | |
| 2 | 3.40 | 2.20 |
| 4 | 3.90 | 2.38 |
| 8 | 3.70 | 2.40 |
| 10 | 3.70 | 2.40 |

a Under conditions described under "Experimental Procedures."

b The first form eluted from affinity chromatography. This particular preparation initially had 3.62 mol of sialic acid per mol of protein.

c The second form eluted from affinity chromatography. This particular preparation had 2.43 mol of sialic acid per mol of protein initially present.
TABLE II

| Isoelectric points of subforms of asialo rabbit plasminogen determined at 22° |
|-----------------------|---------------|---------------|
| Subform               | pI ± 0.1      |               |
|                       | Asialo F-1    | Asialo F-2    |
| 1                     | 8.51          | 8.54          |
| 2                     | 8.91          | 8.97          |
| 3                     | 9.14          | 9.19          |

* The number of the peak in Fig. 3.
* The asialo form of the first affinity chromatography peak.
* The asialo form of the second affinity chromatography peak.

Fig. 2. Polyacrylamide gels, pH 4.3, of mixtures of the rabbit plasminogen affinity chromatography forms. Left, a mixture of native plasminogen forms; right, a mixture of the asialo plasminogen forms.

Fig. 3. Isoelectric focusing profiles of asialo rabbit plasminogen on a pH 3 to 10 gradient at 4°. Top, asialo rabbit plasminogen F-1; bottom, asialo rabbit plasminogen F-2. The experimentally determined pH gradient and the absorbance at 280 nm are presented on the graph.

Fig. 4. Affinity chromatography of the asialo rabbit plasminogen forms at 23°. ○, neuraminidase-treated rabbit plasminogen F-1 and F-2; ●, native rabbit plasminogen F-1 and F-2 shown for comparison. The linear gradient of ε-aminocaproic acid (ε-AHX) used to elute the peaks is shown on the graph.
This manuscript deals with the role of sialic acid in determining the distinct characteristics of the many forms of plasminogen found in the animal. First, two major forms of plasminogen can be resolved based on their differential affinity for antifibrinolytic amino acids of the ε-aminocaproic acid class (1). We wish to propose at this time that these two major forms be classified as isozymes. Although we know essentially nothing concerning their site or sites of synthesis we do know that these forms possess certain physical differences such as their charge characteristics (2), sialic acid content (3), and general states of glycosylation (3), and certain functional differences such as their binding constants to antifibrinolytic acids (2). Metabolic studies show differences in their rates of synthesis and degradation and also show that these forms of plasminogen are not interconvertible in the animal (4). We do not mean to infer that the above mentioned structural and functional differences are the only differences found in these forms but any others remain to be established. It is important to note that removal of the sialic acid from each isozyme of rabbit plasminogen appears to greatly reduce and possibly eliminate the charge differences between these proteins. On the other hand, the only functional difference that we have so far observed between the two isozymes, i.e. their differential binding to ε-aminocaproic acid-like compounds is not affected by this treatment. This is reflected by the identity in affinity chromatography elution profiles of the isozymes of native and asialo rabbit plasminogen. These observations tend to suggest that the charge differences in the rabbit plasminogen isozymes are incidental to their classification as isozymes.

No definitive statements can be made concerning which, if any, of the subforms resolved from each isozyme can be considered to be isozymes. It appears clear that there are small differences in the sialic acid content of the subforms of each isozyme. The possibility that these small sialic acid differences

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**Table III**

| Protein                     | \( s_{0,0}^{20} \pm 0.05 \text{ S} \) |
|-----------------------------|----------------------------------------|
| F-1, native                 | 5.74                                   |
| F-1, native + \( \varepsilon \)-aminocaproic acid | 4.85                                   |
| F-1, asialo                 | 5.71                                   |
| F-1, asialo + \( \varepsilon \)-aminocaproic acid | 4.90                                   |
| F-2, native                 | 5.77                                   |
| F-2, native + \( \varepsilon \)-aminocaproic acid | 4.81                                   |
| F-2, asialo                 | 5.75                                   |
| F-2, asialo + \( \varepsilon \)-aminocaproic acid | 4.88                                   |

* The buffer is 0.1 M Tris-HCl, pH 7.8.

| Protein                     | \( s_{0,0}^{20} \pm 0.05 \text{ S} \) |
|-----------------------------|----------------------------------------|
| F-1, native                 | 5.74                                   |
| F-1, native + \( \varepsilon \)-aminocaproic acid | 4.85                                   |
| F-1, asialo                 | 5.71                                   |
| F-1, asialo + \( \varepsilon \)-aminocaproic acid | 4.90                                   |
| F-2, native                 | 5.77                                   |
| F-2, native + \( \varepsilon \)-aminocaproic acid | 4.81                                   |
| F-2, asialo                 | 5.75                                   |
| F-2, asialo + \( \varepsilon \)-aminocaproic acid | 4.88                                   |

* The buffer is 0.1 M Tris-HCl-0.1 M \( \varepsilon \)-aminocaproic acid, pH 7.8.

**Table IV**

| Subform | Native p\( \varepsilon \) | Sialic acid |
|---------|--------------------------|-------------|
| F-1 (1) | 6.20                      | 3.81        |
| (2)     | 6.55                      | 3.70        |
| (3)     | 6.85                      | 3.57        |
| (4)     | 7.24                      | 3.42        |
| (5)     | 7.78                      | 3.00        |
| F-2 (1) | 6.95                      | 2.67        |
| (2)     | 7.18                      | 2.33        |
| (3)     | 7.89                      | 2.07        |
| (4)     | 8.42                      | 2.06        |
| (5)     | 8.74                      | 1.85        |

* Taken from Reference 2.
in the subforms, which could arise from partial desialylation of the isozymes, could be responsible for the isoelectric focusing separation of the subforms is strengthened by the observation that removal of all sialic acid from each isozyme leads to a loss of resolution of two to three subforms in the isoelectric focusing experiments. We are performing further metabolic studies in order to determine whether any of the subforms did arise by effects which occurred after the individual isozymes were completely synthesized.

Finally, we have found that removal of the sialic acid from each plasminogen isozyme does alter its circulatory survival time under heterologous conditions. However, the lowering of the half-life which accompanies this treatment is not as dramatic as it appears to be for proteins such as ceruloplasmin (5, 6), prothrombin (11), fetuin (6), orosomucoid (6), and others. This difference in behavior of plasminogen cannot be further explained at this time.

In conclusion, we feel that the carbohydrate and particularly the sialic acid plays an important role in defining some of the multiplicity which has been found in rabbit plasminogen. This system is particularly well suited for structural and metabolic studies on this aspect and we are vigorously continuing our efforts in these directions.

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J. Biol. Chem. 1974, 249:7742-7746.

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