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Microheterogeneity of rat α_1-fetoprotein and immunochemical properties

Akiharu WATANABE, Kazuhisa TAKETA
and Kiyowo KOSAKA

The First Department of Internal Medicine, Okayama University Medical School, Okayama, Japan

ABSTRACT

A purified and homogeneous preparation of rat α_1-fetoprotein (α_1-FP), as judged by both electrophoresis on Cellogel and immunoelectrophoresis, were separated into two components, namely α_{1a} and α_{1b}-FP, by disc electrophoresis on 7% polyacrylamide gel. These two components had definite differences in electrostatic net charge and gave only a single band on SDS-disc electrophoresis. Immunological reactivity or electrophoretic separation or mobility thereof could be altered by treatment with either sulfhydryl inhibitors or reducting agents but not by treatment with protein denaturants. Electrophoresis of neuraminidase-treated α_1-FP on 5% polyacrylamide gel yielded clearly separable, slower moving components, first four to six and finally two components depending on the time of incubation with neuraminidase. The time-dependent conversion of faster into slower migrating components of both α_{1a}-FP (R_{BPB} 0.88→0.85→0.83) and α_{1b}-FP (R_{BPB} 0.85→0.80→0.78→0.76) upon neuraminidase treatment was confirmed by re-electrophoresis of separated and similarly treated α_{1a} and α_{1b}-FP. Both α_{1a} and α_{1b}-FP treated with and without neuraminidase gave a single fused precipitin line against the antiserum in Ouchterlony double-diffusion analysis. On the basis of the changes in electrophoretic mobilities of these intermediates, α_{1a} and α_{1b}-FP were estimated to have at least 2.5 and 4.5 molecules of sialic acid per molecule, respectively. The nature of additional negative charges required for α_{1a}-FP to move faster than α_{1b}-FP at an alkaline pH is discussed.

INTRODUCTION

During the course of a biochemical study on the relationship between rat fetal and cancer α_1-fetoprotein (α_1-FP) carried out to investigate the

mechanism of α_1-FP production by liver injuries, we recently found microheterogeneous forms of purified rat α_1-FP on conventional polyacrylamide gel electrophoresis. In the present report, some physicochemical and immunological properties of microheterogeneous forms of α_1-FP were examined in an attempt to elucidate the grounds for the observed microheterogeneity.

MATERIALS AND METHODS

Purification of α_1-FP: α_1-FP was purified from pooled fetal serum by using an immunoadsorbent column of Sepharose coupled with rabbit antibody to rat α_1-FP by means of BrCN[1]. An antibody rich γ-globulin fraction was isolated from specific anti-α_1-FP serum[2] by ammonium sulfate fractionation and lyophilized. α_1-FP fractionated from fetal serum by 43% saturation of ammonium sulfate was applied to the column coupled with the antibody. The eluted preparation from the column was further purified by gel filtration on Sephadex G-200 column which was equilibrated with 5 mM phosphate-buffered saline, pH 7.0.

Analytical techniques: Vertical disc electrophoresis on polyacrylamide gel was performed at 4°C for 70 min with a constant current of 4 mA per tube by the method of Davis[3] as previously described in detail[4].

Neuraminidase treatment of α_1-FP: Sufficient amounts of neuraminidase with a ratio of 0.64 unit enzyme/mg α_1-FP were used at a concentration of 0.5 mg/ml neuraminidase. Incubation of α_1-FP at 37°C with neuraminidase was performed for as long as 12 hr. When α_1-FP was treated with neuraminidase for 12 hr, a further necessary amount (20 munits) of enzyme was added after 6 hr of incubation.

Materials: Neuraminidase (type VI, chromatographically purified from *Clostridium perfringens*) was purchased from Sigma Chemical Co. Other reagents used were purchased from sources reported previously[5].

RESULTS

Electrophoretic properties of the purified α_1-FP: The purified α_1-FP was demonstrated to be homogeneous by both electrophoresis on Cellogel and immunoelectrophoresis. Two components of the purified preparation were, however, revealed by the standard procedure of disc electrophoresis. They had relative mobilities against bromophenol blue (BPB) as a marker, R_{BPB}, of 0.58 and 0.55, the relative amount being 35 and 65% of the total protein, respectively. Fast and slow migrating components were referred to as α_{1a}-FP and α_{1b}-FP.

Physicochemical and immunological properties of α_{1a} and α_{1b}-FP: By disc

electrophoresis with varying concentrations of acrylamide monomer, these two forms were demonstrated to have a difference in net charge but not in molecular size. This was further confirmed by the fact that the purified α_1-FP containing two components gave only a single band corresponding to an approximate molecular weight of 74,000 on SDS-disc electrophoresis.

The purified α_1-FP treated with 0.5 M β-mercaptoethanol failed to react with the antiserum and showed a broad band with slower migration (R_{BPB} of 0.38–0.40). The reactivity of fetoproteins treated with 33 mM $HgCl_2$ or 33 mM N-ethylmaleimide against the anti-α_1-FP serum remained unchanged but the separation of α_{1a} and α_{1b}-FP was obscured to yield one broad band with R_{BPB} of 0.57–0.60. In contrast to these findings, no alteration of electrophoretical and immunological properties of α_1-FP was observed in treatment with 7.5 M urea or 0.33 M guanidine-HCl (Table 1).

Table 1. *Effect of various compounds on immunological and electrophoretic properties of α_{1a} and α_{1b}-FP*.*

Addition (mM)	Immunological reactivity of α_1-FP	Disc electrophoretic pattern (− +)
None	present	two bands near anode
Urea (7.5×10^3)	present	two bands near anode
Guanidine-HCl (330)	present	two bands near anode
$HgCl_2$ (33)	present	one broad band near anode
N-Ethylmaleimide (33)	present	one broad band near anode
β-Mercaptoethanol (500)	absent	one broad band near cathode

* Each 40 μg of the purified α_1-FP was incubated with the indicated concentrations of various compounds at 37°C for 2 hr in a final volume of 90 μl. Immediately after incubation, each aliquot of 45 μl was applied on 7% polyacrylamide gel column and another aliquot of the treated supernatants was analyzed by double-diffusion analyses.

Effect of neuraminidase treatment on electrophoretical properties and immunological reactivity of α_1-FP against the antiserum: Disc electrophoretic pattern of α_1-FP treated for 6 hr with neuraminidase revealed a much more clearly separated two bands with slower mobilities (mean R_{BPB} values of α_{1a} and α_{1b}-FP, 0.53 and 0.48); each of these bands appeared to be further composed of subcomponents. On the basis of the relative amounts of α_{1a} and α_{1b}-FP with or without neuraminidase treatment, these two forms of α_1-FP appeared not to be interconvertible under these conditions.

Disc electrophoresis with 5% acrylamide monomer was thus employed in order to further demonstrate the heterogeneity of neuraminidase-treated α_1-FP. This procedure yielded new components first four (R_{BPB}; 0.85, 0.83, 0.78 and 0.76) to six (R_{BPB}; 0.88, 0.85, 0.83, 0.80, 0.78 and 0.76) and finally two (R_{BPB}; 0.83 and 0.76) depending on the time of incubation with neuraminidase, and untreated α_1-FP were separated into two bands with R_{BPB} of 0.88 and 0.85 (Fig. 1). The time-dependent conversion of faster into slower migrating components of both α_{1a}-FP (R_{BPB}; $0.88 \rightarrow 0.85 \rightarrow 0.83$) and

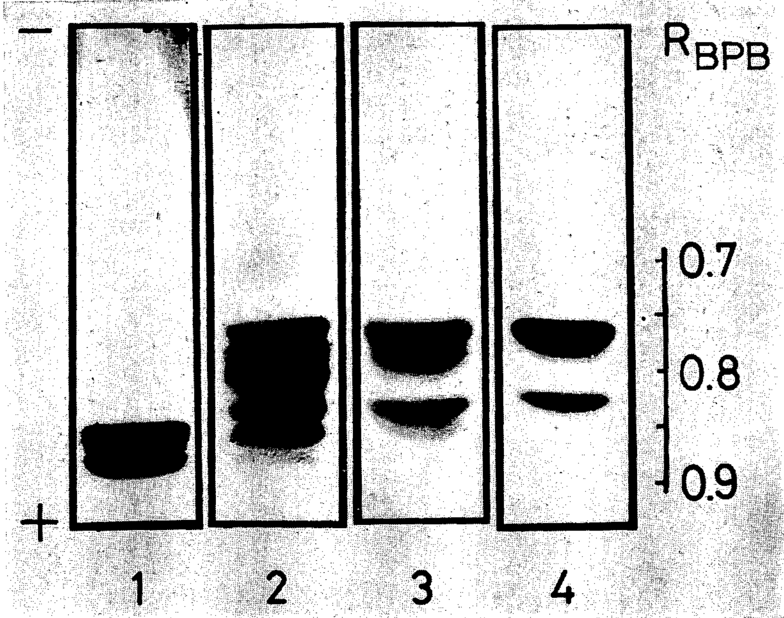

Fig. 1. Disc electrophoretic pattern of α_1-FP treated for various periods of time with neuraminidase. The purified α_1-FP (20 μg) was treated for 6 min (2), 6 hr (3) and 12 hr (4) with neuraminidase as described in Materials and Methods. Treated α_1-FP was electrophoresed on 5% polyacrylamide gels. Electrophoretic pattern of untreated α_1-FP (1) was shown as the control. Neuraminidase was electrophoresed much more faster than any components of α_1-FP under these conditions.

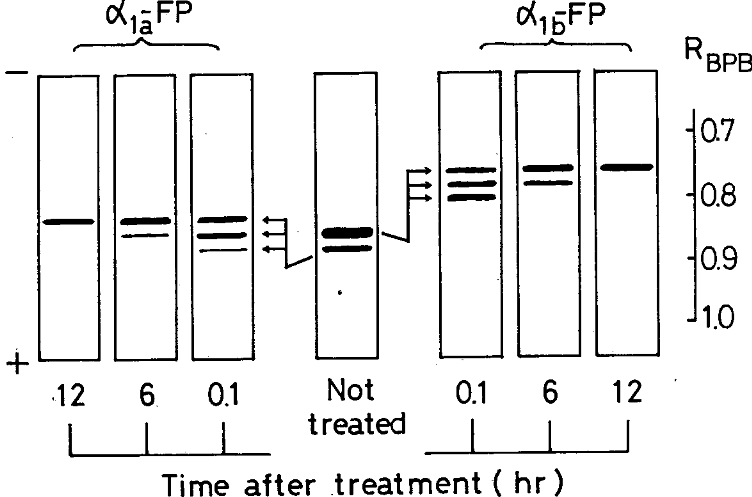

Fig. 2. Effect of neuraminidase treatment on disc electrophoretic mobilities of separated α_{1a} and α_{1b}-FP. Forty μg of α_1-FP was electrophoresed on 5% polyacrylamide gels. α_{1a} and α_{1b}-FP were separately cut from gels with respect to R_{BPB} values of each component, minced and extracted at 37°C with mechanical stirring in a small volume of 50 mM Tris-HCl, pH 7.5. The extracted solutions from identical gels were pooled, concentrated by ultrafiltration through a collodion membrane and immediately treated with neuraminidase for the indicated periods of time. Re-electrophoresis of treated α_{1a} and α_{1b}-FP was performed similarly on 5% polyacrylamide gels. A schematic illustration of disc electrophoretic patterns is shown.

α_{1b}-FP (R_{BPB}; $0.85 \rightarrow 0.80 \rightarrow 0.78 \rightarrow 0.76$) upon neuraminidase treatment was confirmed by re-electrophoresis of separated and neuraminidase-treated α_{1a} and α_{1b}-FP (Fig. 2). As in the above no interconversion of the two forms was seen in this observation either.

α_{1a} and α_{1b}-FP treated with or without neuraminidase gave a single fused precipitin line against the antiserum in Ouchterlony double-diffusion analysis. The distinct immunoelectrophoretic separation of α_{1a} and α_{1b}-FP treated for 12 hr with neuraminidase was also demonstrated on the agarose plate. A plot of assumed numbers of sialic acid groups removed *vs.* distance travelled of α_1-FP components suggests that at least 2.5 and 4.5 molecules of sialic acid per mole are to be removed to explain the transformation of α_{1a}-FP with R_{BPB} of 0.88 and α_{1b}-FP with R_{BPB} of 0.85 into α_{1a}-FP with R_{BPB} of 0.83 and α_{1b}-FP with R_{BPB} of 0.76 upon treatment with neuraminidase.

DISCUSSION

The changes in electrophoretic mobility, conversion of faster into slower migrating components of both α_{1a}-FP (R_{BPB} 0.88→0.83) and α_{1b}-FP (R_{BPB} 0.85→0.76), which occured after treatment with neuraminidase suggest a stepwise removal of sialic acid residues from each component. The intermediate forms found during relatively short incubation with neuraminidase would represent the elimination of at least 1 (α_{1a}-FP) and 1, 2 and 3.5 (α_{1b}-FP) molecules of sialic acid in the decreasing order of electrophoretic mobility. Similar observations in which successive removal of sialic acid gave a sequence of progressively slower moving components on electrophoresis have been reported for cases of microheterogeneous forms of several proteins[6,7].

Treatment of fetuin[7] and human α_1-FP[8] with neuraminidase reduced the number of electrophoretic bands, indicating that sialic acid is primarily responsible for the observed microheterogeneity. However, the removal of sialic acid from rat α_{1a} and α_{1b}-FP in contrast revealed a more clear separation of these fetoproteins. Furthermore, two protein bands of sialized or desialized α_1-FP were still observed on electrofocusing (unpublished observation). These results suggest that additional negative charges required for α_{1a}-FP to move faster than α_{1b}-FP at an alkaline pH on disc electrophoresis is donated by a dissociable group(s) with a pK_a above the isoelectric points.

Use of sulfhydryl inhibitors and reducing agents such as β-mercaptoethanol reveals that some protein structures (the conformation of the molecule) must be maintained to give two distinct electrophoretic bands. Unlike fetuin[7], however, α_1-FP can be similarly separated by electrophoresis into two components even in the presence of urea as may be the case for α_1-glycoprotein[9]. Thus, there is a possibility that the existence of two α_1-FP may originate from subtle differences unaffected by urea treatment in the number or arrangement of amide groups or of disulfide cross linkages or in the carbohydrate portion of the molecule. This would also offer further potential for variation or conformational differences that influence the arrangement of the charged groups at the surface of a molecule.

Proof of this must await sufficient preparations of pure samples of α_{1a} and α_{1b}-FP for analyses to be meaningful.

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