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Research Article

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The α_1-Acid Glycoprotein Variants of Normal Caucasian and Japanese Individuals *

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Recent investigations of the polymorphism of α_1-acid glycoprotein demonstrated that this blood globulin may resolve on starch gel electrophoresis in five, six, seven, or eight bands depending on the individual from whom the glycoprotein was derived (1). However, it was noted earlier that pooled α_1-acid glycoprotein from which the sialic acid had been split off enzymatically exhibited only two main bands (2). This simple pattern suggested the presence of α_1-acid glycoprotein variants in individual blood and, thus, the possibility of elucidating the genetic transmission of and the chemical differences between the variants of this plasma protein.

In this paper the starch gel electrophoretic analysis of sialic acid-free α_1-acid glycoprotein preparations derived from blood of normal Caucasian and Japanese individuals is presented.

Methods

Ninety-seven apparently healthy white adults and nineteen pairs of Caucasian twins from the Boston area donated blood for this study. Ten of these pairs of twins were shown to be nonidentical as judged by thirteen blood groups. The probability that the "identical" twins were fraternal was calculated to be 0.01. Sixty-four sera from apparently healthy Japanese whose ages ranged from 20 to 40 years were collected in the Sendai and Tokyo areas of Japan.

Neuraminidase (*Vibrio cholerae*) was obtained commercially.[1] One ml of the enzyme solution contained 100 U of enzyme activity (1 U splits 1 μg of sialic acid from glycopeptides at 37° C in 15 minutes). This enzyme was free of proteolytic activity. A highly purified and extremely concentrated *Clostridium perfringens* neuraminidase was generously donated.[2] The latter preparation was approximately 4,000 times more active than the former.

The preparation of the partially separated, polymorphic forms of native α_1-acid glycoprotein derived from pooled Caucasian blood was described earlier (3).

The fractionation of the sera was carried out according to an abbreviated modification of Cohn's method 10 (1). Because this technique was developed for the fractionation of plasma it proved advantageous to add to the serum specimens the required amount of acid-citrate-dextrose solution (4), namely, 2.0 ml of this solution per 23 ml of serum. Subsequently α_1-acid glycoprotein was isolated from Fraction VI by chromatography on Amberlite IRC-50 (5). As demonstrated earlier (1) such preparations appear homogeneous on paper electrophoresis (6), ultracentrifugation, and starch gel electrophoresis (7) from pH 4 to 9.

For the removal of sialic acid, the native α_1-acid glycoprotein preparations were incubated with neuraminidase. Free sialic acid was determined by the method of Warren (8). The digest was dialyzed exhaustively against cold distilled water and lyophilized. Bound sialic acid was measured by the same procedure after hydrolysis at 80° C with 0.10 or 0.2 N sulfuric acid for 1 hour without subsequent removal of the latter acid.

The horizontal starch gel electrophoresis was carried out according to Smithies (7) using pH 5.0, ionic strength 0.02 acetate buffer (when measured at room temperature). Mixing the buffer with the required amount of partially hydrolyzed starch powder raised the pH almost to 5.1. The starch gel block was $24 \times 10 \times 0.5$ cm. Two mg of enzymatically modified glycoprotein was employed for each analysis (1, 3). The starch gel electrophoresis was carried out for 6 hours in a 2° cold room at 270 v (13 v per cm) yielding 60 to 70 ma. Under these conditions the slowest moving band moved about 5 mm from the trough toward the anode. During the latter part of this study 2.0 mg of modified glycoprotein was dissolved in 0.01 ml of the mentioned acetate buffer and

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[1] Behringwerke, Marburg a./L., Germany.

[2] Supplied by Drs. S. Roseman and W. G. Jourdian, University of Michigan, Ann Arbor, Mich.

adsorbed on a piece of Whatman 3 MM paper (10 mm × 5 mm), which was then inserted into the starch gel in the position where earlier the troughs were cut out. Amido black was used to stain the gels. The staining solution contained 100 ml of glacial acetic acid, 500 ml of ethanol, 500 ml of water, and 1 g of dye. The destaining solution had the same composition except that it was free of dye. The relative intensities of the protein bands were derived from densitometer measurements of negatives of the patterns employing a microdensitometer [3] (1) or by visual comparison with an appropriate standard.

A Spinco model E analytical ultracentrifuge equipped with an automatic rotor temperature indicator controller unit (R.T.I.C. type) and schlieren optics were used for the characterization of the modified protein.

In preliminary digestion experiments, pooled α_1-acid glycoprotein was incubated for different periods of time to establish the optimal condition for removal of the sialic acid. Twenty-five mg of pooled α_1-acid glycoprotein was dissolved in 5 ml of neuraminidase solution [1] and incubated at 37° C. The same amount of enzyme solution was added after 2 and 4 hours. Samples containing 2.5 mg of protein were removed at the incubation times indicated in Figure 1, dialyzed,[4] lyophilized, analyzed for sialic acid, and finally subjected to starch gel electrophoresis at pH 5.1. In another series of experiments 2.5 mg of glycoprotein was incubated with 0.1, 0.25, 0.5, and 1.0 ml of enzyme solution, respectively, at 37° C for 12 hours.

For the enzymatic digestion of the individual α_1-acid glycoprotein preparations, 2.5 mg of α_1-acid glycoprotein was dissolved in 0.5 ml of the Behring enzyme solution and incubated at 37° C for 24 hours. After 2 and 4 hours, another 0.5 ml of the enzyme preparation was added. Subsequently, the digests were dialyzed at 2° C against several changes of cold distilled water and lyophilized. No bacteriostatics were added to the digest during the incubation time.

Results

A. Digestion of pooled α_1-acid glycoprotein with neuraminidase. The starch gel electrophoretic analysis of pH 5.1 of pooled α_1-acid glycoprotein previously incubated with a constant amount of neuraminidase for varying periods of time revealed, first, a decreasing number of bands and, second, a change in the relative intensity especially of the slower moving bands as the digestion proceeded (Figure 1). Two main bands and a faster moving minor band were noted after 24 hours. A trace component with an even higher apparent electrophoretic mobility was often observed. The latter exhibited the highest apparent electrophoretic mobility. The average relative percentages of the color intensity of the two main bands were determined to be 45 and 50%, respectively. No further change in the pattern was found when the incubation time was prolonged for an additional 24 hours. If the enzymatic digestion was incomplete, the relative color intensity of the two faster components was relatively high. It could be shown by chemical analysis of the protein eluted from these zones that any sialic acid present in such digests is associated with these faster moving components. Untreated pooled α_1-acid glycoprotein exhibited a single band at this pH. The determination of the released and bound sialic acid of α_1-acid glycoprotein shows that the bulk of this acid (92%) is cleaved off in 2 hours and that it takes an additional 22 hours and further enzyme to split off another 4%. With the highly active *Clostridium*

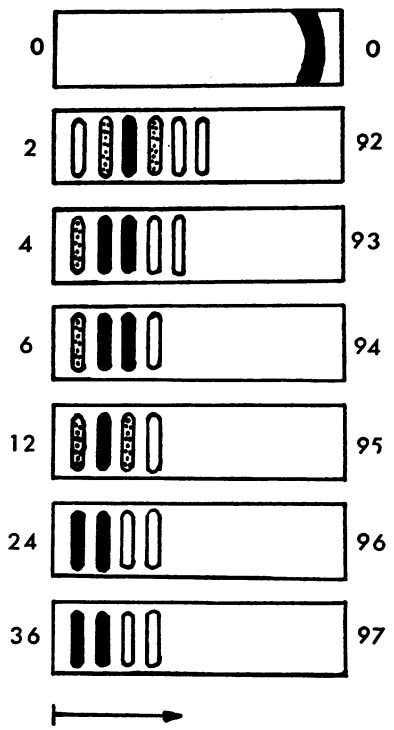

FIG. 1. STARCH GEL ELECTROPHORETIC ANALYSIS AT pH 5.1 OF POOLED α_1-ACID GLYCOPROTEIN DIGESTED WITH NEURAMINIDASE AT 37° FOR DIFFERENT PERIODS OF TIME AS INDICATED IN HOURS ON THE LEFT OF THE PATTERNS. The percentage of sialic acid removed is indicated on the right of the patterns. The direction of migration of the protein is indicated by the arrow.

[3] Model E12, M, Goyce, Loebel & Co., Ltd., Maidenhead on Tyne, England.

[4] The outside solution of the dialysis process was analyzed for free amino acids using the dinitrophenyl (DNP) procedure of Sanger (9). All DNP-derivatives behaved like dinitroaniline (9) demonstrating the absence of proteolytic enzymes in the neuraminidase preparations.

perfringens enzyme preparation it was possible to hydrolyze more than 99% of the sialic acid. The starch gel pattern of the latter preparation did not reveal a fourth fastest moving trace component, and its third fastest moving component accounted only for a very small percentage of the total color.

The results of the starch gel electrophoretic analysis of the α_1-acid glycoprotein digested with varying amounts of enzyme for a constant period of 12 hours at 37° C indicated that 1.0 ml of enzyme per 2.5 mg of glycoprotein was not sufficient to obtain maximal removal of sialic acid and that 1.5 ml of enzyme and an incubation time of 24 hours were required.

As control, starch gel electrophoretic analysis was performed on 5 ml of enzyme solution, previously kept for 24 hours at 37° C and concentrated by lyophilization after dialysis. The obtained pattern did not reveal any stainable material.

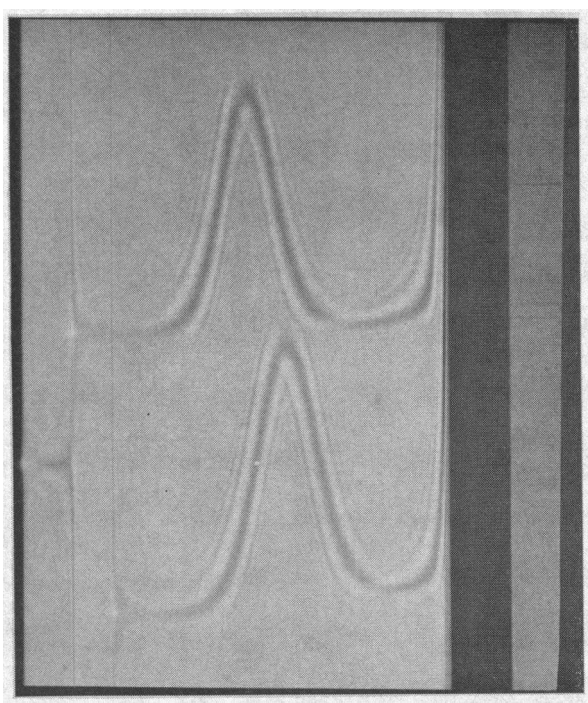

FIG. 2. ULTRACENTRIFUGAL ANALYSIS OF SIALIC ACID-FREE (UPPER CURVES) AND ACTIVE (LOWER CURVES) α_1-ACID GLYCOPROTEIN DERIVED FROM POOLED NORMAL HUMAN PLASMA. The picture was taken after 140 minutes of revolution at 56,200 rpm. The direction of sedimenting is from left to right. Note that the distance between the two menisci and that between the maxima of the two refractive index curves are the same at any time during this analysis. Hence, both proteins sediment at the same rate.

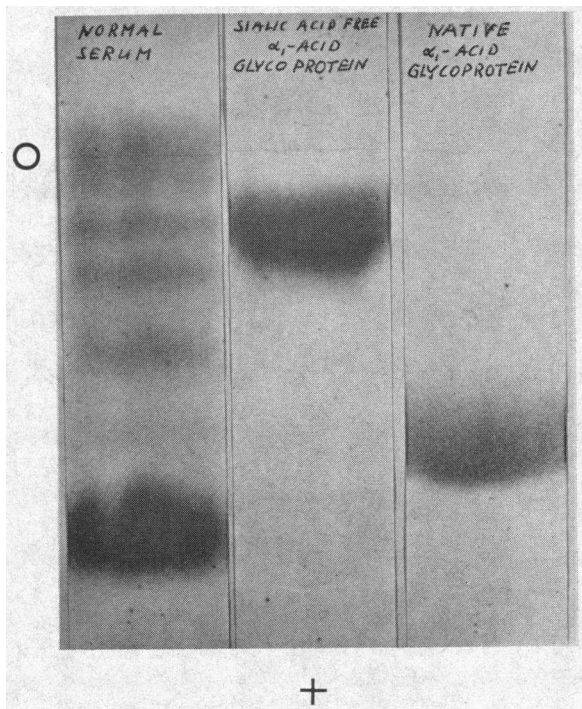

FIG. 3. PAPER ELECTROPHORETIC ANALYSIS OF SIALIC ACID-FREE α_1-ACID GLYCOPROTEIN AT pH 8.6 IN $\Gamma/2$ 0.2 CITRATE BARBITURATE BUFFER (ELECTROPHORETOGRAM IN CENTER). As reference the native form of this protein and normal human plasma were utilized. The protein solutions were applied at the lines designated with (O). The anodic side of the paper strips is designated with +. Electrophoresis was carried out at a voltage of 80 v corresponding to 2.7 v per cm for 17 hours.

B. Characterization of the neuraminidase-treated pooled α_1-acid glycoprotein. Ultracentrifugal analysis of pooled α_1-acid glycoprotein from which 96% of its sialic acid had been removed enzymatically was carried out in pH 7.6, ionic strength 0.1 phosphate buffer simultaneously with untreated α_1-acid glycoprotein, both at a concentration of 1%. The obtained schlieren pattern (Figure 2) indicated homogeneity. The sedimentation coefficient ($s^{1\%}_{20,w}$) was calculated to be 2.7 S for both preparations indicating that this glycoprotein was not digested by proteolytic enzymes possibly present in the neuraminidase preparation. Further, the refractive index gradient curves (Figure 2) were essentially identical suggesting that the diffusion coefficient and thus the shape of both the native and the modified protein are very similar. Moreover, as judged by paper chromatography, the absence of free amino acids or peptides in the dialyzate of the sialidase-digested α_1-acid glycoprotein

demonstrated clearly the absence of proteolytic enzymes in these enzyme preparations.

Paper electrophoresis of the modified and native glycoprotein at pH 8.6 in ionic strength 0.1 citrate-diethylbarbiturate buffer revealed homogeneity (Figure 3). The electrophoretic mobility at pH 8.6 of these two preparations corresponded to that of the β_1- and α_1-globulin (10), respectively.

Starch gel electrophoretic analysis of several sialic acid-free pooled α_1-acid glycoprotein preparations have been analyzed about fifty times and always yielded the same pattern, indicating that this procedure is highly reproducible.

Although all starch gel electrophoretic analyses of the modified α_1-acid glycoprotein isolated from individual blood were carried out at pH 5.1, an additional series of starch gel electrophoreses using pooled, modified α_1-acid glycoprotein was performed as a function of pH keeping the ionic strength constant at 0.02. Between pH 4.6 and 5.4 excellent separation of the two bands was observed, and the relative percentage of the color was found to be the same. As expected, the apparent electrophoretic mobility changed as a function of pH. At pH 5.7 and 4.3 the two bands, of which the electrophoretic mobilities were very high, barely separated.

Immunoelectrophoretic analysis (11, 12) of the modified and native α_1-acid glycoprotein using a rabbit serum active against pooled normal α_1-acid glycoprotein revealed identity. As judged by this criterion these results indicated that the variants have certain immunochemical determinants in common.

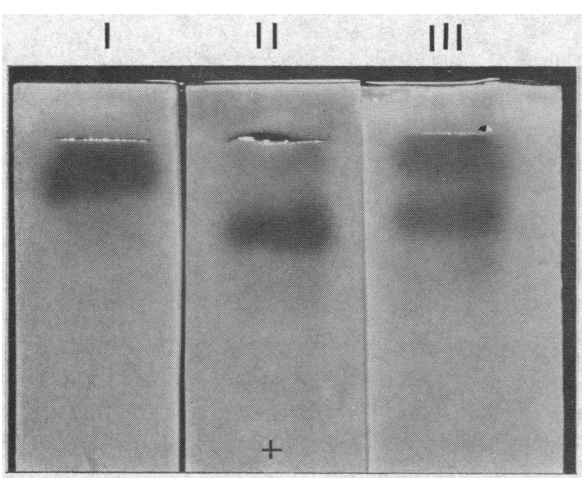

FIG. 4. THE THREE TYPES OF SIALIC ACID-FREE α_1-ACID GLYCOPROTEIN PATTERNS (I, II, III). Starch gel electrophoresis was performed in pH 5.1, $\Gamma/2$ 0.02 acetate buffer at 270 v for 6 hours. Under these conditions the proteins migrated toward the anode +.

TABLE I

Relative color intensity of the protein bands present in patterns of the three types of α_1-acid glycoprotein variants

Type	Relative percentage of color intensity of the protein bands Band number 1*	2	3	4
I	80	15	5	+†
II	15	80	5	+
III	45	50	5	+

* Slowest moving band.
† + = trace.

C. Analysis of sialic acid-free α_1-acid glycoprotein preparations derived from normal Caucasian and Japanese individuals. Three types of patterns were observed (Figure 4). Type I is characterized by one slow moving main and a faster moving minor band and type II by a slow moving minor and one faster moving main band. Type III shows 2 main bands with apparent electrophoretic mobilities corresponding to those of the main bands of types I and II. The relative color intensity of the observed bands of these three types is given in Table I. All patterns of type I showed approximately 15% of a protein with an apparent electrophoretic mobility corresponding to that of the main component of type II and vice versa. Each pattern usually showed two additional faster moving minor components of which the relative color intensity appeared to be constant. The protein present in these two bands was found to be incompletely digested glycoprotein.

Additional rare patterns were observed: In a very small number of the type II patterns the slowest moving band accounted for 25 to 30% of the total color. In a small number of type I patterns the second band was increased in color to 25 to 30%. Nevertheless, in the present study these patterns were included in the respective groups. Studies are in progress to determine whether these patterns represent atypical types of variants.

Ninety-seven sialic acid-free α_1-acid glycoprotein preparations derived from normal white adults revealed patterns of the above described three types of which the relative percentage was 11 : 46 :

43 (Table II). Sixty-four glycoprotein preparations from normal Japanese adults yielded patterns of the same types. The relative frequency of the three types differing from those of Caucasian origin was found to be 17 : 63 : 20 (Table II).

The analysis of the corresponding preparations from nineteen pairs of twins is given in Table III. Each of the identical twins showed the same pattern. Four pairs of the fraternal twins each revealed different patterns (pair no. 5, 7, 15, and 17), whereas the other pairs of fraternal twins each showed the same patterns. However, the chi-square test applied to this relatively small number of analyses did not indicate a significant difference between the two groups.[5]

D. Relationship between the relative percentage of polymorphic forms and that of the variants of α_1-acid glycoprotein derived from Caucasian normal adults. The relationship between α_1-acid glycoprotein patterns observed at pH 5.1 and those noted at pH 2.9 (Table IV) is not clear at present. There appears to be a trend in that type II and type III are primarily associated with pH 2.9 patterns with 6 and 7 bands. No conclusions can be drawn with regard to type I and patterns with 5 or 8 bands whose frequency of occurrence was too low. The complexity of the relationship between the pH 2.9 and 5.1 starch gel electrophoretic patterns suggests that different properties are measured with the two types of analyses. This is demonstrated by the comparison of the data of three pairs of fraternal twins that yielded the same patterns at pH 5.1 but different ones at pH 2.9.

TABLE II

*Relative percentage of the three α_1-acid glycoprotein patterns in Caucasian and Japanese normal adults**

Type	Caucasian		Japanese	
	Number of analyses	Relative %	Number of analyses	Relative %
I	11	11	11	17
II	45	46	40	63
III	41	43	13	20

* Neuraminidase treated α_1-acid glycoprotein preparations analyzed at pH 5.1

[5] A convenient nomogram giving the relationship among chi-square, degrees of freedom, and p is found in: Boyd, W. C., A nomogram for chi-square. J. Amer. Statistical Ass. 1965, **60**, 344.

TABLE III

Starch gel electrophoretic analysis of α_1-acid glycoprotein derived from nineteen pairs of twins

Twins		Analysis at pH 5.1 of sialic acid-free α_1-acid glycoprotein Type of pattern	Analysis at pH 2.9 of native α_1-acid glycoprotein (1) Number of bands observed in pattern
Pair	Zygosity*		
		no.	
1a	i	III	6
1b		III	6
2a	i	III	6
2b		III	6
3a	i	III	7
3b		III	7
4a	i	III	7
4b		III	7
5a	f	I	6
5b		III	6
6a	f	III	7
6b		III	7
7a	f	III	6
7b		II	7
8a	f	II	6
8b		II	7
9a	i	II	6
9b		II	6
10a	f	II	7
10b		II	7
11a	f	II	7
11b		II	8
12a	i	I	8
12b		I	8
13a	i	III	7
13b		III	7
14a	i	III	7
14b		III	7
15a	f	II	6
15b	i	III	6
15c		III	6
16a	f	II	7
16b		II	6
17a	f	II	6
17b		III	†
18a	f	II	6
18b		II	6

* i = identical; f = fraternal. This characterization is based on the results obtained from typing 13 different blood groups.
† Analysis failed.

The patterns of other fraternal twins show the reverse (Table III).

E. Analysis of the α_1-acid glycoprotein variants partially separated in their native form. The partially separated polymorphic forms of native, pooled α_1-acid glycoprotein (3), after digestion with neuraminidase, were analyzed by starch gel electrophoresis under the same conditions. The obtained results summarized in Table IV indicated the following shift in the patterns of sialic acid-free protein preparations: The relative percentage of the two main bands changed gradually from a ratio of 55 : 40 (similar to type III), found for

Fraction 2, to 7:85 (similar to type II), noted for Fraction 9.

Discussion

A reduction in the number of bands of low molecular weight plasma glycoproteins observed on starch gel electrophoresis due to enzymatic removal of its sialic acid has been reported earlier for β_2-mucoid (13, 14), haptoglobins (15), and transferrin (16) and recently for α_1-acid glycoprotein (2) and Zn- and Ba-α_2-glycoproteins (17–19). In contrast, the starch gel electrophoretic patterns of the different cholinesterases do not change upon digestion with neuraminidase (20, 21).

A further point of interest of this investigation is that the cleavage of sialyl residues from human plasma glycoproteins by bacterial neuraminidases derived either from *Vibrio cholerae* or *Clostridium perfringens* will cleave off over 90% of this acid within a very short period of incubation time, but for essentially complete removal, a significantly longer incubation time and additional enzyme are required. It would be of great interest to learn whether a sialidase derived from a mammalian source that might be characterized by a different specificity would split off sialic acid residues at a much higher rate. The observed rate of cleavage of sialic acid may be a reflection of the finding by Jeanloz and Closse (22) that the sialic acid residues of α_1-acid glycoprotein are bound to three different carbon atoms of galactose. It is possible that one of these three linkages is relatively difficult to be cleaved by the mentioned bacterial neuraminidases.

The main finding of this study is that α_1-acid glycoprotein isolated from blood of normal white adults and treated with neuraminidase yielded 3 types of patterns on starch gel electrophoresis at pH 5.1, e.g., near the isoelectric point of this modified glycoprotein. Above pH 6 and below 4 homogeneity was observed under otherwise identical conditions. The analysis of the modified α_1-acid glycoprotein yielding simple patterns that are obtained at significantly more neutral pH values than that of the native α_1-acid glycoprotein (pH 4.8 vs. pH 2.9) lends further assurance of the presence of the variants of α_1-acid glycoprotein. No difficulties were encountered in classifying the pH 5 patterns in contrast to the corresponding relatively complex patterns of the unmodified α_1-acid glycoproteins that require concomitant control runs of a standard for their evaluation. Based on the results of a study on a series of families it will be possible to indicate which types are homozygotes and which are heterozygotes.

The relative percentage of the three types of patterns among the white population (Boston area) appears significantly different from that of the Japanese. Type I is relatively rare in both populations. Types II and III are observed in Caucasian people at almost the same frequency, whereas type II seems to occur among Japanese at a higher frequency than among the Boston Caucasian adults and type III shows the reverse.

TABLE IV

Relationship between the patterns of native and sialic acid-free α_1-acid glycoprotein preparations derived from blood of normal Caucasian adults

Starch gel electrophoretic analysis at pH 2.9 (native)		Starch gel electrophoretic analysis at pH 5.1 (modified) number of cases with type		
Number of bands in pattern	Number of cases	I	II	III
5	1	0	1	0
6	24	2	13	9
7	25	2	11	12
8	7	1	3	3

TABLE V

Starch gel electrophoretic analysis of the α_1-acid glycoprotein variants partially separated in their native form by chromatography on a DEAE-cellulose column

Properties of partially separated, native α_1-acid glycoprotein variants					
		Analysis at pH 5.1 of sialic acid-free fractions (Relative percentage of color intensity) Band number			
Fraction*	Isoionic point†	1‡	2	3	Type of pattern
2	3.54	55	40	5	III
3		55	40	5	
4		45	50	5	
5	Gradual	40	55	5	Gradual
6	change	35	60	5	change
7		35	60	5	
8		10	80	10	
9	3.32	7	85	8	II

* The number of zones observed on starch gel electrophoresis at pH 2.9, their mobilities, and their relative percentage are described in reference 3.
† Taken from reference 3.
‡ Protein band with lowest apparent electrophoretic mobility.

Larger series of analyses are needed to establish the exact differences between the relative distribution of the three types of patterns for both investigated populations. In addition, studies are in order to establish whether or not a relationship exists with regard to sex.

The possible presence of hereditary variants was suggested by the study of α_1-acid glycoprotein derived from twins. Each pair of "identical" twins was always characterized by identical patterns. As expected, a certain number of fraternal twins yielded different patterns. Further indication that the α_1-acid glycoprotein variants may be genetically determined is derived from the significantly different ratio of the three types of patterns observed in the Caucasian and Japanese populations.

With regard to the physical chemical or perhaps chemical difference of the α_1-acid glycoprotein variants, a relationship was observed between the isoelectric point of the fractions obtained by partial separation of pooled normal α_1-acid glycoprotein (3) and the starch gel electrophoretic patterns at pH 5 of these fractions after their treatment with neuraminidase. Type III pattern was associated with the most neutral fraction (isoionic point 3.54) and type II with the most acidic fraction (isoionic point 3.32) (1). A gradual parallel change of these two parameters, isoionic point and pH 5 patterns of the fractions, was observed as a function of the acidity of the original fractions. Although type I does not occur at a high frequency in the Caucasian population and does not appear in this series of analyses, it may be said that chemically type III might be considered as the sum of types I and II. Thus, type I must be comparatively enriched in the relatively neutral fractions and hence must be more neutral than type II, even in its native, i.e., sialic acid carrying, forms. This conclusion was confirmed by the analysis of the sialic acid-free forms of these two types. Therefore, the fundamental difference between the two α_1-acid glycoprotein variants cannot be related to their apparently equal sialic acid content (3) and probably must be associated with the amino acid composition of their polypeptide moieties.

In an attempt to explain in a preliminary and oversimplified fashion the formation of the complex, polymorphic individual native glycoproteins from their simple, sialic acid-free variants, the following observations of others are considered. It is known that the pK of sialyl residues bound to different carbon atoms of galactosyl residues vary significantly (23). In addition, Jeanloz and Closse (22) showed that in pooled Caucasian α_1-acid glycoprotein sialic acid is linked in approximately equal number to carbon numbers 3, 4, and 6, and Jourdian (24) demonstrated the existence of two sialyl transferases that preferentially transfer the sialyl residues to carbon numbers 3 or 6 of galactose, respectively. As pointed out above, types I, II, and III patterns were each associated essentially with an equal number of pH 2.9 patterns with 6 and 7 bands. From these findings it might be speculated that the sialyl residues of each α_1-acid glycoprotein variant are distributed among the C-atoms of galactose so as to yield patterns with 5, 6, 7, or 8 bands. Thus, whereas the difference between the variants is perhaps associated with the polypeptide moiety of the glycoprotein, the polymorphic patterns of the native forms appear to be related to the type and relative activity of sialyl transferases the individual produces. Consequently, it may be further speculated that such transferases in turn may also be genetically determined in order to form the apparently genetically determined polymorphic forms of α_1-acid glycoprotein.

Summary

α_1-Acid glycoprotein was isolated from blood of apparently healthy Caucasian adults, including nineteen pairs of twins, and sixty-four Japanese adults. Sialic acid was cleaved off with neuraminidase, and the resulting modified protein was analyzed by starch gel electrophoresis at pH 5.1.

Three types of patterns were obtained. The relative percentage of these patterns was determined for Caucasian and Japanese adults to be 12:46:43 and 17:62:21, respectively. Each pair of "identical" twins always revealed the same type of pattern, whereas four of the ten pairs of fraternal twins each showed different patterns. It was suggested that the different patterns may represent genetically determined variants of α_1-acid glycoprotein and that the fundamental difference between these variants is probably associated with the polypeptide moiety of the protein.

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