ACTION OF α-GALACTOSIDASE ON GLYCOPROTEIN FROM HUMAN B-ERYTHROCYTES

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SUMMARY A soluble glycoprotein fraction extracted by butanol-water treatment of human blood-group B erythrocytes showed strong B-activity. Treatment of this glycoprotein with α-galactosidase extracted from coffee beans caused the disappearance of its B-activity with the concomitant liberation of D-galactose as the only low-molecular weight saccharide. Terminal, non-reducing, α-linked D-galactose is known to be essential for the activity of water-soluble glycoproteins isolated from body fluids on which most research on blood-group substances has hitherto been concentrated.

Soluble blood-group substances (glycoproteins) isolated from a variety of body fluids are under active investigation in a number of laboratories, and much information has already been obtained on their chemical structure and biochemistry[1-3]. Carbohydrate moieties have been implicated as their immunological determinants.

Isolation and study of the antigens present on erythrocyte surfaces (the blood group determinants) has been much less successful, and it is not clear at present whether the substances involved are glycoproteins or glycolipids[4-6]. Both glycolipids[4, 6] and glycoproteins[5, 6] with blood-group A, B, H activity have been isolated from human erythrocytes under a variety of conditions. The soluble glycoprotein with blood-group B activity has an immunodominant α-D-galactopyranosyl linkage at the non-reducing terminal[2], but no information is available concerning the chemical structure of proteins with similar immunological properties present in erythrocytes. It was considered of interest, therefore, to isolate a glycoprotein fraction with B-activity from human erythrocytes and to study its structure and properties. As a first approach, it was decided to follow the effect of α-galactosidase on its composition and immunological properties. We showed that a glycoprotein

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fraction was extracted by butanol-water treatment from the stroma of blood-group B erythrocytes which did, indeed, have strong B-activity; on treatment of this glycoprotein with coffee bean α-galactosidase, the B-activity disappeared with the concomitant liberation of D-galactose.

MATERIALS

Reagents All chemical reagents and solvents were of the highest grade of purity available. p-Nitrophenyl-N-acetyl-β-D-galactosaminide was purchased from Pierce Chemical Co., Rockford, Ill., while the p-nitrophenyl glycopyranosides of D-glucose (α and β), D-galactose (α and β), L-fucose (α), D-mannose (α) and N-acetyl-D-glucosamine (β) were purchased from Koch-Light Laboratories, Colnbrook, Bucks., England. Anti-A and anti-B sera were obtained from DADE-Division, American Hospital Supply Corporation, Miami, Florida. Human erythrocytes from healthy individuals of blood groups A and B were isolated from outdated blood donated by the blood bank of Tel Hashomer Hospital, Israel.

Buffers The buffers employed were:

1) 0.06M NaCl, 0.025M Na_2HPO_4, 0.008M KH_2PO_4, 0.001M EDTA, pH 7.2
2) 0.02M NaCl, 0.01M Na_2HPO_4-KH_2PO_4, 0.001M EDTA, pH 7.2
3) 0.01M NaCl, 0.005M Na_2HPO_4-KH_2PO_4, 0.001M EDTA, pH 7.2
4) 0.01M sodium acetate, pH 7.0
5) 0.1M sodium acetate, pH 5.0
6) 0.15M NaCl, 0.01M Na_2HPO_4-NaH_2PO_4, pH 7.4

METHODS

Preparation of Stroma Erythrocyte membranes were prepared from human B-type blood by an adaptation of the methods of Danon, et.al.[7] and Rega et.al.[8]. The blood was centrifuged at 1,750 x g for 10 min (International Centrifuge) and the plasma and buffy coat removed by aspiration. The cells were washed thrice with 0.17M NaCl solution followed by re-centrifugation at 1,750 x g. The washed erythrocytes were resuspended at a hematocrit of 2.5% in buffer 1 and dialysed against buffer 2 (30 x volume) for 3 h. This procedure was performed twice. After centrifugation at 10,000 x g for 20 min at 4^0 (Sorvall, Model B), the precipitated ghosts were washed four times with buffer 3 and thrice with deionized, distilled water and isolated by centrifugation at 14,500 x g at 4^0 for 20 min.

Paper Chromatographic Analysis The descending method on Whatman No. 1 paper was employed with the following solvent mixtures: A, 4:5:1 butanol-acetic acid-water; B, 6:4:3 butanol-pyridine-water. Sugars were revealed by treatment with

the silver nitrate dip reagent[9] and aminosugars by ninhydrin spray[10].

Thin-layer Chromatography Micro-slides were coated with silica gel G (Merck and Co., Darmstadt) and the plates were developed in solvents A, B or C (4:5:1 butanol-ethanol-water). Spots were revealed by charring with sulfuric acid.

Proteins were determined by the method of Lowry, et. al.[11]. Hexoses were analyzed with phenol-sulfuric acid[12] against D-galactose as standard and reducing sugars by the Park-Johnson method[13].

Isolation of Glycoprotein from Membranes Stroma were extracted with aqueous butanol[6]. The aqueous phase was concentrated in vacuo, $T < 35^0$, with several additions of water to remove the dissolved butanol. The residue dissolved completely in water or acetate buffer (pH 5-7). (Lyophilization of the aqueous solution generally resulted in a soluble residue, but occasionally a powder remained which was only partially soluble in water or acetate buffer.) In a typical preparation, 50 mg of glycoprotein (equivalent to 36 mg of albumin - Lowry) was obtained from 80 ml of packed erythrocytes. The sample contained 2 mg hexose, and paper chromatography (solvents A, B) revealed the presence of small amounts of migrating saccharides.

A solution of 125 mg of this material (collected from several preparations) in 1.5 ml of buffer 4 was applied to a column of Sephadex G-50 (95 x 2.5 cm). Fractions of 4 ml were collected and examined for absorption at 220 nm and 280 nm, total hexose and protein. A peak emerged with the void volume which comprised 90-95% of the material added to the column. Hexose analysis showed this peak to contain 2.5-3.0% hexose (25-30 γ/mg). It was free from migrating saccharides (2 days paper chromatography), including galactose.

Isolation of α-Galactosidase Coffee beans were extracted with 0.17M NaCl solution, and the crude extract purified as described by Courtois and Petek[14]. The peak designated α_1 which emerged from the alumina column was used as the enzymic preparation for the degradation of the glycoprotein. It catalysed the hydrolysis of p-nitrophenyl-α-D-galactopyranoside but had no effect on the corresponding β-D-galactopyranoside nor on the p-nitrophenyl glycopyranosides of D-mannose(α), D-glucose (α or β), N-acetyl-D-glucosamine (β) and N-acetyl-D-galactosamine (β).

Enzymic Degradation of the Glycoprotein A mixture of glycoprotein and enzyme (10:1 ratio according to protein analysis) was incubated in buffer 5 at 37^0. Maximum liberation of reducing sugar was attained after 72 h. The mixture was boiled for 5 min and centrifuged for 10 min at 4,000 x g. The clear supernatant was added to

a small column of 1:1 charcoal-celite 545 and eluted with a large volume of water until the eluate was free of dissolved salts and sugars. The eluate was concentrated to a small volume, passed through a column of Amberlite 1R120(H^+) and reconcentrated. Aliquots were examined by t.l.c. and paper chromatography and showed the presence of galactose as the sole sugar. A sample of 60 mg of glycoprotein, treated in this way, afforded 45 γ of reducing sugar - estimated as galactose - in the water-eluate from the charcoal column. Reaction of the eluate with galactose oxidase gave 42 γ of D-galactose (i.e. 0.7 γ/mg of the original glycoprotein).

A sample of 25 mg of the glycoprotein, incubated with boiled enzyme for three days and then treated as above, gave no apparent release of galactose, and the water-eluate from the charcoal column afforded no reaction with galactose oxidase and did not contain any reducing sugar.

In both experiments - enzymic hydrolysis and control - elution of the charcoal column with an aqueous ethanol gradient after the water elution (up to 25% ethanol) did not lead to the appearance of any carbohydrate material.

Hemagglutination Reaction Microhemagglutination assays were performed on disposable microtiter plates[15] (Cooke Engineering Co., Alexandria, Va.). All test solutions were in buffer 6, pH 7.4. The protein concentrations[11] were adjusted to 2.5 mg/ml. Serial two-fold dilutions of the antisera were made in buffer 6. One volume of test-material or of buffer 6 was added to one volume of antiserum and the solution incubated at 37° for 1 h. One volume of a 2% suspension of washed red cells in buffer 6 was added, the mixture was kept at ambient temperature for 2 h and the agglutination was read, numbers being given for the extent of inhibition of hemagglutination (Table I).

RESULTS

Treatment of erythrocyte stroma from human B-blood with aqueous butanol resulted in the extraction of more than 80% of the protein into the aqueous phase. The partially purified glycoprotein emerged as a single peak from a column of Sephadex G-50, even though previous workers have shown that it is not homogeneous[8]. It contained 2.5-3.0% of hexose and was free of lipid. It reacted strongly with anti-B serum and gave no reaction with anti-A. Partially purified α-galactosidase, isolated from coffee beans, catalysed the hydrolysis of α-D-galactopyranosides but showed no other glycosidase activity. Incubation of the glycoprotein with this enzyme preparation led to almost complete disappearance of B-activity with the concomitant liberation of 2.5-3.0% of the total hexose present in the original sample as D-galactose. Disaccharides and other derivatives of galactose were not produced.

Table I: Hemagglutination Inhibition Activity of Glycoprotein from B-Erythrocytes

Sample	Erythrocyte Type	Dilutions of sera 1:1	1:2	1:4	1:8	1:32	1:64	1:128
		Anti-B						
Buffer 6	B	+1	0	0	0	0	0	0
Buffer 6 + glycoprotein	B	+4	+4	+4	+4	+4	+4	+4
Buffer 6 + enzyme-treated glycoprotein	B	+2	+2	+1	0	0	0	0
		Anti-A						
Buffer 6 + glycoprotein	A	0	0	0	0	0	0	0

CONCLUSION

The blood group-B activity of a glycoprotein isolated from human erythrocytes which contains most of the original protein present in the stroma was removed by enzymic treatment. At the same time, D-galactose was liberated. This finding is in agreement with the known necessity of the α-D-galactopyranosyl moiety for the blood group-B activity of soluble blood group substances[1]. Examination of the effect of similar treatment on glycolipids extracted from erythrocytes and on whole red cells may give an indication of the types of compounds involved in the specific immunochemical reactions of erythrocytes, their structures and their location in the cell-membrane.

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