COMPOSITION OF BLEPHARMONE, A CONJUGATION-INDUCING GLYCOPROTEIN OF THE CILIATE *BLEPHARISMA*

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1. Introduction

For conjugation between complementary mating types of the ciliate *Blepharisma intermedium,* two gamones are necessary [1]. Type I cells excrete gamone 1, which transforms type II cells so that they can unite. Type II cells excrete gamone 2, which similarly transforms type I cells. Each gamone promotes the production of the other gamone. Gamone 2, called blepharismone, was identified as calcium-3-(2′-formylamino-5′-hydroxybenzoyl) lactate [2]. Gamone 1, designated as blepharmone, was isolated and tentatively characterized as a glycoprotein [3].

The high mol. wt of blepharmone of about 20 000 [3,4], inactivation by heat, proteases and 8 M urea, and in addition its staining reactions, led to the conclusion that blepharmone might be a glycoprotein. We report here on the amino acid and the sugar composition of blepharmone which proves that it is a glycoprotein. The glycoprotein contains unusually high proportions of certain amino acids especially those which serve as sugar attachment sites.

2. Materials and methods

Blepharmone was obtained by incubating type I cells with blepharismone as described [3,4]. Synthetic blepharismone [5] was used after purifying as indicated [2]. Blepharmone was purified by subsequent passages through columns of Bio-Gel P-150, carboxymethylcellulose and diethylaminoethylcellulose as outlined previously [3]. In this way 3.64 mg of blepharmone with the average activity 1.6×10^7 units/mg was prepared. One unit activity is defined as the least amount of blepharmone that can induce homotypic cell union in 10^3 cells of type II suspended in 1 ml buffer [4].

Electrophoresis on 9% or 12% polyacrylamide gel slabs in the presence of 0.1% sodium dodecylsulfate (SDS) was done as follows: 20 μg of protein in 20 μl buffer (0.1% mercaptoethanol, 0.001 M EDTA, 0.001 sodium phosphate, pH 7.0) was mixed with 2 μl 50% glycerol containing 0.05% Bromphenol Blue and applied to the gel. Electrophoresis was performed in the apparatus Ge-4 of Pharmacia at constant current (18 mA) overnight with the buffer 0.01 M sodium phosphate, 0.005 M EDTA, 0.1% SDS in the electrode chamber. Amino acids and amino sugars were determined with an Unichrom amino acid analyser (Beckman, Munich) using a two-column system. Addition of 4% methanol to the routine starting buffer for the analysis of the neutral and acidic amino acids resulted in a complete separation of aspartate, methionine sulfone, threonine and serine. Glucosamine was determined both as the peak before lysine and the peak after phenylalanine. Mannosamine and galactosamine are eluted later than glucosamine. Cysteine and methionine were oxidized with performic acid [6] to cysteic acid and methionine sulfone and determined as such. Samples were hydrolysed under nitrogen either with 4 N HCl for 15 hr at 105°C or with 5.7 N HCl (constant boiling HCl) for 22 hr at 105°C.

For the determination of neutral sugars 0.688 mg glycoprotein was hydrolysed in 0.9 ml 0.01 N HCl in the presence of 200 mg Dowex 50 X 8, 200–400

mesh in the H^+- form for 40 hr at 100°C. The hydrolysate was neutralized with the ion exchange resin IRA 410, HCO_3^--form. The aldoses thus formed were converted into alditol acetates [7] which were determined with a gas-liquid chromatograph (Varian Aerograph model 1520 B) on a glass column (0.32 X 152 cm) filled with ECNSS-M (3% on Gaschrom Q, 100/120 mesh) at a column temp. of 170°C and a nitrogen flow rate of 30 ml/min. Known amounts of xylose and rhamnose were added as standards for quantitation. Sialic acid was determined with the thiobarbituric acid assay after hydrolysis of the glycoprotein in 0.1 N H_2SO_4 at 80°C for 1 hr [10]. A standard curve obtained with 2-16 μg sialic acid (Serva, Heidelberg) permitted quantitation.

3. Results and discussion

3.1. *Determination of molecular weight*

The isolation procedure of blepharmone includes gel chromatography on Bio-Gel P-150. It was deduced from the elution position that blepharmone has a mol. wt of about 20 000. This size agreed with the retention of blepharmone by Amicon-PM 10 membranes and its passage through PM 30 membranes when it was filtered under pressure [3]. To test the purity of the blepharmone sample under investigation and to obtain a more accurate mol. wt for calculation of the number of amino acid residues in the polypeptide chain we ran samples on polyacrylamide gel slabs together with standard proteins in the presence of SDS. We obtained in 9% and 12% polyacrylamide gels only one band with an apparent mol. wt of 30 000 (fig.1) instead of the expected 20 000. It is known for many glycoproteins that their electrophoretic mobility is lower than expected considering their true size, thus counterfeiting a higher mol. wt [9]. But the mobility of many glycoproteins resembles more closely the true mol. wt in higher cross-linked gels than in less cross-linked gels. The mobility of blepharmone however is too small in both gels to enable one to ascribe to it the tentative mol. wt of 20 000. The reason for this is unknown since the carbohydrate content is low (see below) and the protein is not acidic (isoelectric point, 7.2). As noticed by others [9] glycoproteins lacking sialic acid often have an unusually low mobility independent of the gel pore size. We confirm these observations with the electrophoretic behaviour of blepharmone.

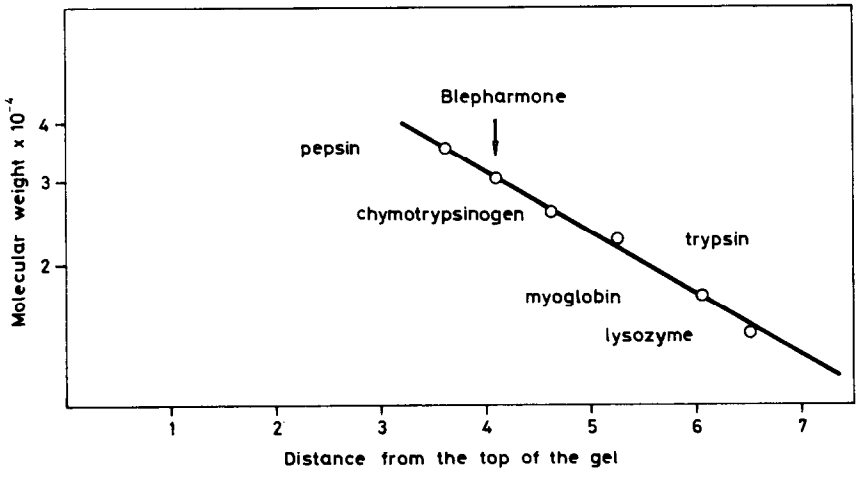

Fig.1. Mol. wt estimation of blepharmone. Electrophoresis of blepharmone on 12% polyacrylamide gel slabs in the presence of 0.1% SDS was performed together with the following standard proteins: pepsin (hog stomach, mol wt 35 000), chymotrypsinogen (bovine pancreas, mol. wt 25 700), trypsin (bovine pancreas, mol. wt 23 300), myoglobin (horse heart, mol. wt 17 200) lysozyme (hen egg white, mol. wt 14 300). The conditions of electrophoresis are described in Materials and methods. The distances of the protein bands from the top of the gel slab were plotted against the logarithm of the mol. wts.

3.2. *Composition of blepharmone*

To determine the amino acid and amino sugar composition of blepharmone, two independently isolated samples were first hydrolysed with 4 N HCl at 105°C for 15 hr to prevent destruction of possible hexosamines. One additional peak besides those of amino acids was noticed at the elution position of glucosamine. Stronger hydrolysis conditions (table 1, column 3) were applied for a more complete release of the hydrophobic amino acids, leucine, isoleucine and valine. The samples were first oxidized with performic acid to convert cysteine into cysteic acid and also for the determination of methionine as methionine sulfone. A tentative amino acid composition in terms of number of residues for each amino acid present in the polypeptide chain was then calculated on the basis that the smallest peak, histidine, corresponded to one residue (table 1, last column). Other than tryptophan, there was a total of 175 amino acids resulting in a mol. wt of 19 000. Tryptophan was not determined due to the lack of enough pure blepharmone. The unusually high content of tyrosine practically excluded the use of material-saving spectrophotometric methods alone for an accurate determination of tryptophan [11]. The very high tyrosine content can be seen from the comparison with 200 proteins listed by G. Reeck [12], of which less than 2% have tyrosine values as high as blepharmone. The values for aspartic acid (or asparagine), threonine and serine are very high too but this is also true of other glycoproteins. These amino acids serve in glycoproteins as attachment sites for the carbohydrate units. The content of glutamic acid in contrast is very low. Thus blepharmone has unique structural features compared to other proteins, and can easily be identified by the amino acid composition in future works.

The analysis of the sugars in blepharmone yielded glucosamine as the sole hexosamine while the only neutral sugar was mannose. Three residues of each sugar are present in the polypeptide chain. No traces of sialic acid could be found. Also treatment with neuraminidase did not affect the biological activity of blepharmone [4]. The sugar content amounts to 5% and the total mol. wt (without tryptophan) adds up to 20 000. Both values are in excellent agreement with earlier, much less accurate determinations in which the sugar content was estimated by staining the protein band on polyacrylamide gels with the periodic acid-Schiff's reagent using orosomucoid and β_2-glycoprotein as standards [3] and in which the mol. wt was deduced from column chromatography on Bio-Gel P-150 [4].

Table 1
Composition of the glycoprotein blepharmone (numbers of amino acids, amino sugars and neutral sugars per polypeptide chain)

Amino acid	Hydrolysis 4 N HCl 15 hr	Hydrolysis 5.7 N HCl 22 hr	Nearest integer
Lysine	6.94	6.87	7
Histidine	0.89	1.00	1
Arginine	4.01	3.84	4
Aspartic acid	25.62	26.70	26
Threonine	15.98	17.33	17
Serine	19.62	17.98	19
Glutamic acid	7.04	7.01	7
Proline	6.74	8.76	8
Glycine	12.92	13.80	13
Alanine	13.49	13.06	13
Cysteine	–[a]	3.87[a]	4
Valine	10.58	10.88	11
Methionine	4.86	5.94[b]	6
Isoleucine	6.87	7.78	8
Leucine	11.33	11.66	12
Tyrosine	13.09	–[c]	13
Phenylalanine	5.96	6.07	6
Tryptophan	–[d]	–[d]	
Glucosamine	2.97	2.99	3
Neutral sugars:			
Mannose	2.2% corresponding to 2.65 residues[e]		3

The figures are mean values of duplicate analyses of two independently isolated samples.

a Cysteine was determined as cysteic acid
b Methionine was determined as methionine sulfone
c Tyrosine was destroyed by performic acid oxidation due to chloride in the sample
d Tryptophan was not determined
e The percentage of mannose is based on the protein content of the sample measured with the Lowry method [8] with bovine serum albumin as standard. Small peaks equal to 10% of the mannose peak were observed on the gas chromatogram at the positions of glucose and fucose

Blepharmone is released from the cell and has to interact with the cell of opposite mating type, presumably first with the cell surface, to induce conjugation [13]. It is tempting to assume that the sugar portion of the protein plays an essential role in the recognition process between blepharmone and cell surface structures. The polypeptide chain of blepharmone has the high polarity [14] of 46% and is easily soluble in water so that binding of blepharmone to the cell membrane might possibly involve carbohydrate interactions rather than hydrophobic parts of the molecule. Carbohydrate specific modification reactions should yield the answer to this problem.

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