STUDIES ON MUCOPROTEINS

II. ANALYSIS OF THE PROTEIN MOIETY OF OVINE SUBMAXILLARY GLAND MUCOPROTEIN

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SUMMARY

Ovine submaxillary gland mucoprotein (OSM), shown previously to have the disaccharide α-D-N-acetylneuraminyl(2→6)N-acetylgalactosamine as prosthetic group, was analysed for its constituent amino acids and its amide content. OSM was found to have an almost complete complement of amino acids though with characteristic features. Its amide-N (0.34 % of total N) could only be determined after enzymic removal of its terminal neuraminic acid residues since even on mild acid hydrolysis the release of amide-N was accompanied by the degradation of N-acetylneuraminic acid with liberation of about half its N as ammonia-N. OSM exhibited a single peak on electrophoretic examination and in the ultracentrifuge.

INTRODUCTION

In the biological test ovine submaxillary gland mucoprotein (OSM) is the most potent of the influenza virus haemagglutinin inhibitory mucoproteins[1]. Chemically OSM is characterized by the presence in it of equimolecular quantities of N-acetylneuraminic acid (NANA) and N-acetylgalactosamine (NAGal) with traces only of other sugars, the latter being regarded as carbohydrate moiety of a contaminating mucoprotein. In a previous paper[2] the isolation, purification and analysis of the prosthetic group was described and its structure shown to be that of the disaccharide 6-α-D-N-acetylneuraminyl-NAGal. The carbohydrate-prosthetic group constitutes 42 % by weight of the mucoprotein molecule.

It seemed desirable to have a qualitative and quantitative analysis of the protein moiety of OSM including its amide content since no complete protein analysis is available for any of the influenza virus haemagglutinin inhibitory mucoproteins. Such an analysis was also required for a more detailed investigation into the type of linkage joining the prosthetic group to the polypeptide chain. Though CURTAIN AND PYE[3] have reported electrophoretic homogeneity for their OSM preparation, a comparison of their material with our OSM is not possible since the former authors have not characterized chemically their preparation. The homogeneity of OSM used in our work was therefore tested by electrophoresis and in the ultracentrifuge.

EXPERIMENTAL

Materials

OSM was prepared by the procedure described previously[2].

The amino acids used as reference substances were crystalline and of analytical grade.

The crystalline D-galactosamine hydrochloride was the preparation described previously[2].

Vibrio cholerae neuraminidase (RDE) was prepared in a highly purified state by Drs. G. ADA and E. FRENCH, Walter and Eliza Hall Institute of Medical Research, Melbourne, and kindly presented by them.

Ion exchange resins used were Dowex I-X2 (50–100 mesh) and Dowex 50-X8; the latter was hydraulically classified by the method of HAMILTON[4], particles 35–70 μ being selected.

Methods

50 mg samples of lyophilised OSM were hydrolysed for 20 and 70 h respectively with 5 ml of twice distilled constant boiling point hydrochloric acid in a sealed tube at 110°. The amino acids in the hydrolysate were separated by ion-exchange chromatography[5] and assayed with the ninhydrin reagent of MOORE AND STEIN[6]. The complete quantitation was carried out in the eight column automatic amino acid analysis equipment described by SIMMONDS AND ROWLANDS[7].

The total dicarboxylic acids in the hydrolysate of OSM (10 *N* HCl, 110°, 24 h) were determined independently by adsorption onto Dowex I-X2 (acetate form) followed by washing with water to remove neutral and basic amino acids and by eluting with acetic acid. The effluent and the eluate were separately lyophilised. The absence of aspartic and glutamic acids in the effluent and their presence uncontaminated by other amino acids in the eluate were verified by paper partition chromatography. The monoaminodicarboxylic acids in the eluate were determined according to MOORE AND STEIN[6].

Total nitrogen of OSM and of the ammonia-N in the 6 *N* HCl hydrolysate after 20 h and 70 h were determined by the micro Kjeldahl method.

Proline was determined in the acid hydrolysate of OSM by paper chromatography using serial dilutions of proline as standards and the isatin reagent[8] as spray; the solvent system was *n*-butanol–pyridine–water (6:4:3, v/v).

Alkaline hydrolysates of OSM (2 *N* NaOH or *N* $Ba(OH)_2$, 100°, 24 h) were tested for tryptophan by paper chromatography using the *p*-dimethylaminobenzaldehyde reagent as a spray.

Ammonia liberated from OSM by 2 *N* and *N* HCl respectively in a sealed tube at 100° for specified times was determined by the micro diffusion method of CONWAY AND O'MALLEY[9].

Total sulphur was determined according to ZIMMERMANN[10].

Electrophoresis was carried out at 2° in a Tiselius apparatus using the 2-ml cell (channel length 5 cm, cross section 0.30 cm^2). Patterns were photographed with a Land camera, a strip tungsten lamp serving as light source. Prior to electrophoresis the OSM solutions were dialysed at 2° for two days against two changes of the buffer used in the experiment.

RESULTS

Electrophoretic examination of OSM

Electrophoresis of OSM (1 % w/v) in phosphate buffer (pH 7.3; ionic strength 0.2) and in veronal buffer (pH 8.6; I = 0.1) revealed a single peak in both the ascending and descending limbs. The mobilities of the mucoprotein in the two buffers were -7.4 and $-8.1 \cdot 10^{-5}$ cm^2 sec^{-1} V^{-1} respectively. The electrophoretic patterns of OSM in veronal buffer at various time intervals are reproduced in Fig. 1. Similarly a single peak only was found on ultracentrifugal analysis. A detailed report on the behaviour of OSM in the ultracentrifuge and on the viscosity of OSM will be given in a subsequent paper.

Main components of OSM

The main components of OSM and their percentage by weight are shown in Table I.

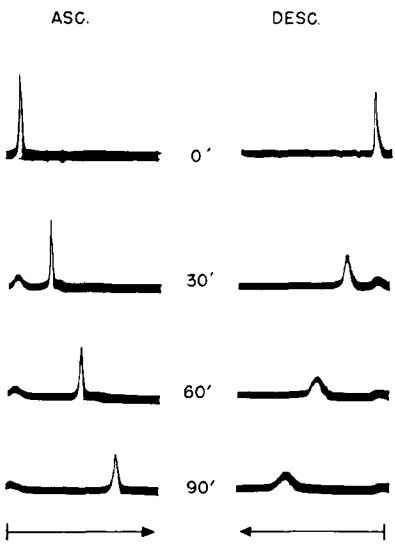

Fig. 1. Electrophoretic pattern of OSM in veronal buffer (pH 8.6; I = 0.1) at a potential gradient of 7.06 V/cm at intervals of 30 min.

TABLE I

ANALYTICAL DATA FOR OSM

	Percent of dry weight
N-acetylneuraminic acid	25.0
N-acetylgalactosamine	18.5
Carbohydrate residue	42.0*
Protein residue	58.0**
Total nitrogen	11.6
Carbohydrate nitrogen	2.3
Protein nitrogen	16.0
Sulphur	0.43

* Allowing for elimination of 1 molecule H_2O between NANA and NAGal.
** By difference.

TABLE II

AMINO ACID ANALYSIS OF OSM

Amino acid	Amino acid nitrogen as % of total nitrogen					
	a 20-h hydrolysis mean*	*b* 70-h hydrolysis mean*	*c* Overall mean or extrapolated value	*d* Standard error	*e* Grams of amino acid per 100 g OSM**	*f* Grams of amino acid residue per 100 g OSM
Alanine	6.67	7.10	6.88	0.14	5.08	4.05
Arginine	9.21	9.14	9.18	0.20	3.31	2.97
Aspartic acid	2.81	2.72	2.76	0.04	3.04 (4.10***)	2.63 (3.55)
Cystine					1.61***	1.37
Glutamic acid	3.54	3.97	3.76	0.10	4.58 (6.17***)	4.02 (5.42)
Glycine	9.38	9.82	9.60	0.16	5.97	4.54
Histidine	0.67	0.72	0.70	0.06	0.30	0.27
Isoleucine		1.75	1.75	0.09	1.90	1.64
Leucine	2.68	2.70	2.69	0.01	2.92	2.52
Lysine	2.40	2.54	2.47	0.11	1.50	1.32
Methionine					0 or trace	
Phenylalanine		1.43	1.43	0.04	1.96	1.75
Proline					5.60***	4.72
Serine	7.76	5.39	8.70	0.13	7.57	6.27
Threonine	6.79	6.14	7.10	0.11	7.00	5.94
Tryptophan					0 or trace	
Tyrosine	0.45	0.45	0.45	0.008	0.68	0.61
Valine	4.45	4.21	4.33	0.10	4.20	3.55
"Amide"	10.49§§	14.90§§				
						48.17 (50.49)
						+ 42.00 carbohydrate
						− 1.48 water§
						88.69 (91.01)

* Mean of three independent analytical runs.

** Calculated according to the formula $\frac{\text{entry of column c} \times 11.6.}{\text{percent N in amino acid}}$

*** Determined by independent method.

§ Allowing for loss of 1 molecule H_2O per prosthetic group-amino acid linkage.

§§ Ammonia-N in percent of total N.

Amino acid analysis of OSM

The results of the quantitative amino acid determinations are summarised in Table II. The absence of tryptophan was indicated by a negative Ehrlich reaction of the chromatogram of the alkali hydrolysate of OSM. The quantity of methionine present was too small to be measured. Though no cystine was detected in the effluent fractions from the ion-exchange columns, its presence in OSM was indicated by an appreciable sulphur content. Since the prosthetic group is free of sulphur, the sulphur content of OSM is expressed in Table II as cystine. The total dicarboxylic acids, as determined by an independent procedure (see METHODS), amounted to 10.7 % expressed as glutamic acid. This quantity was proportionated to aspartic and glutamic acids in the ratio obtained with the MOORE AND STEIN procedure; the resulting figures are given in brackets (Table II).

Amide determination in OSM

When OSM was heated in *N* HCl at 100° for various periods of time and the

ammonia-N released determined quantitatively, the values shown in Fig. 2 were obtained. However, even under these mild conditions of hydrolysis the NANA component of OSM decomposed with humin formation, as was already observed previously[11]. To eliminate any possible contribution of the decomposing NANA to the ammonia formed, OSM was digested with neuraminidase prior to acid treatment.

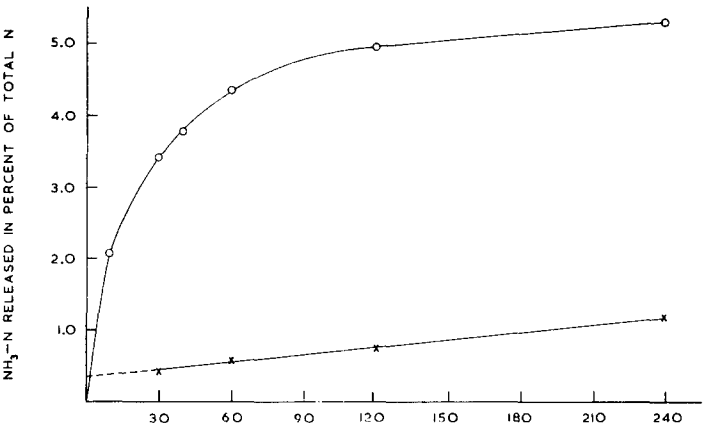

Fig. 2. Time curve of the formation of ammonia-N from 5.0 mg OSM on treatment with N HCl at 100°; O—O, before; ×—×, after release of NANA by digestion with 10,000 units RDE at 35° for 6 h.

The ammonia-N values after removal of 80 % NANA and corrected for the remaining 20 % are included in Fig. 2. Hydrolysis at 100° with 2 N HCl instead of N HCl prior to ammonia determination gave practically the same results. Linear regressions were fitted to the experimental figures by the method of least squares and estimates made of the intercept with the ordinate. The linear limb of the OSM curve extrapolates to 4.49 % N (referred to total N), the OSM-RDE curve to 0.34 % N. The slope of the latter curve is +0.2073 per hour and statistically indistinguishable from the final slope of the OSM curve. The intercept of the OSM-RDE curve, *i.e.* 0.34 % N referred to total N, is taken as amide-N following the procedure of LEACH AND PARKHILL[12].

DISCUSSION

The protein moiety of OSM has an almost complete complement of amino acids; however, no appreciable amounts of tryptophan and methionine seem to be present. Further characteristic features of the protein are its low content of tyrosine, lysine and histidine, its relatively high content of proline and its high content of serine threonine and glycine.

Of the 11.3 (9.0) % mucoprotein not accounted for in Table II, column f, one part was degraded with the formation of ammonia during acid hydrolysis preceding the MOORE AND STEIN fractionation and determination of the constituent amino acids. Thus, of the 10.49 % ammonia-N (referred to total N) present in the 20-h hydrolysate only 4.49 % was derived from NANA and from amide, whereas 6 % of the total N equal to 0.696 g N or 0.696 × 100/16 = 4.35 g amino acid residues/100 g OSM were deaminated in the hydrolysis procedure (6 N HCl, 100°, 20 h). Included

in this figure is part at least of the 2.30 g residues of serine (0.67 g), threonine (0.26 g) and cystine (1.37 g) whose destruction is already allowed for in Table II, column f. The remaining deficit must be due to loss of amino acids by irreversible condensation with sugar constituents[13,14] of OSM and/or in the process of column chromatography. Losses in the determination of the sugar components are allowed for in the carbohydrate figures given (see GRAHAM AND GOTTSCHALK[2]). A complete balance sheet of the constituents of a mucoprotein rich in carbohydrate seems at present not feasible for the reasons discussed above. WEIMER *et al.*[15] analysing the acidic mucoprotein (orosomucoid) of human plasma could account only for 86 % of the total weight.

The somewhat higher values of dicarboxylic acids in the independent determination (Table II) may be due to the application of more severe hydrolysis conditions than those used in the MOORE AND STEIN procedure. Some support for this interpretation may be seen in the increase by 12.1 % in the glutamic acid value when the hydrolysis time was extended from 20 h to 70 h. Perhaps a fraction of the dicarboxylic acids is engaged in a peptide linkage rather resistant to acid hydrolysis, a situation well known for some glycosidic linkages in mixed oligo- or polysaccharides containing free amino groups.

As is evident from Fig. 2, determination of amide-N in sialic acid containing mucoproteins is practicable only after enzymic removal of the terminal sialic acid residues. Once removed the amide-N may be conveniently determined according to the linear extrapolation procedure described by LEACH AND PARKHILL[12]. The amide-N of OSM is very low.

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