Volume 228, number 2, 317-320 FEB 05586 February 1988

Asparaginyl-rhamnose: a novel type of protein-carbohydrate linkage in a eubacterial surface-layer glycoprotein

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Received 26 November 1987; revised version received 25 December 1987

The subunits of the crystalline surface layer of *Bacillus stearothermophilus*, strain NRS 2004/3a contain carbohydrates covalently linked to protein. Hydrolysis of a glycopeptide obtained by pronase digestion of the glycoprotein and analysis of the fragments revealed that rhamnose is *N*-glycosidically linked to the amide nitrogen of an asparaginyl residue.

Eubacteria; S-layer; Glycoprotein; Cell surface; *N*-Glycan

1. INTRODUCTION

The protein subunits of the crystalline surface layers (S-layers) frequently found on eubacteria [1] are glycosylated in certain strains [2–4]. Proteolytic degradation of the S-layer of *Bacillus stearothermophilus* NRS 2004/3a has yielded two glycopeptides, designated GP I and GP II, whose glycan structures have been elucidated [5,6]. The glycan of GP I consists of trisaccharide repeats of rhamnose, whereas in GP II tetrasaccharide repeats of di-*N*-acetylmannuronic acids, *N*-acetylglucosamine and glucose are found. Here, we report on the nature of the protein-carbohydrate linkage present in GP I.

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Part of this work was presented at the 9th International Symposium on Glycoconjugates, Lille, France, July 6–11, 1987

Abbreviations: S-layer, surface-layer; GP, glycopeptide; GC, gas-liquid chromatography; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

2. MATERIALS AND METHODS

2.1. *Sample preparation*

Growth of *B. stearothermophilus* strain NRS 2004/3a, isolation and purification of the S-layer material, and preparation of the glycopeptides were performed as described [6,7].

2.2. *Analytical methods*

Hexoses and rhamnose were determined colorimetrically as in [7]. Monosaccharides were analysed by GC as their alditol acetates in a Packard model 437A gas chromatograph on a Chrompack CP-SIL 8 CB capillary column (30 m; running condition: temperature gradient from 160 to 215°C at 2°C/min) after hydrolysis of the sample in 4 M trifluoroacetic acid at 100°C for 4 h. The protein content was estimated by the method of Lowry et al. [8] with ovalbumin as standard. Amino acids were quantified either colorimetrically with ninhydrin as in [9] or after hydrolysis of the samples in 4 M hydrochloric acid at 100°C for 4 h or 4 M trifluoroacetic acid at 100°C for 4–8 h on a Microtechna model T339 amino acid analyser.

2.3. *Other methods*

SDS-PAGE was performed according to Laemmli [10] on 8% slab gels [11] and preparative SDS-PAGE of intact S-layer glycoprotein was performed as in [12].

TLC on silica gel 60 plates (Merck) was done in butanol/acetic acid/water (80:20:30, v/v) and evaluated by spraying with orcinol [0.2 mg/ml in 20% (v/v) sulfuric acid] or ninhydrin (1 mg/ml in ethanol) reagents.

2.4. *Hydrogen fluoride treatment*

Anhydrous HF [13] was condensed into liquid nitrogen-

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00145793/88/$3.50

cooled reaction vials and melted in an ice bath according to Wieland et al. [14]. The acid was quickly added to the lyophilized S-layer glycoprotein or glycopeptide (0.15 mg/ml). After incubation of the closed teflon vial at 0°C for 1 h with stirring, the reaction was terminated by removing the HF with a gentle stream of nitrogen.

The deglycosylated protein was thoroughly dialysed against 10 mM ammonium bicarbonate buffer (pH 7.8) and analysed by SDS-PAGE after lyophilization.

After HF treatment of the glycopeptide, the reaction products were neutralized with solid ammonium bicarbonate and separated on a calibrated Bio-Gel P-2 column (1 × 113 cm) with 0.1 mM NaCl as the eluant. The fractions (1 ml) were tested for rhamnose colorimetrically and for amino acids by TLC (ninhydrin reagent). Appropriate fractions were concentrated, equilibrated in 0.04 M ammonium bicarbonate buffer, pH 7.8, and applied to a DEAE Bio-Gel A column (1.6 × 5.5 cm; equilibrated in the same buffer). After washing the column with 5 bed volumes of starting buffer the sample was eluted with a linear gradient (2 × 50 ml) of 0.04–0.5 M ammonium bicarbonate buffer, pH 7.8. The rhamnose content of the fractions (1 ml) was estimated. Fractions of interest were collected, concentrated and rechromatographed on the Bio-Gel P-2 column (1 × 113 cm). After concentration of the rhamnose-positive fractions to ~1 ml the remaining solution was divided into two equal portions which, after hydrolysis with 4 M trifluoroacetic acid, were subjected to the GC and amino acid analyses.

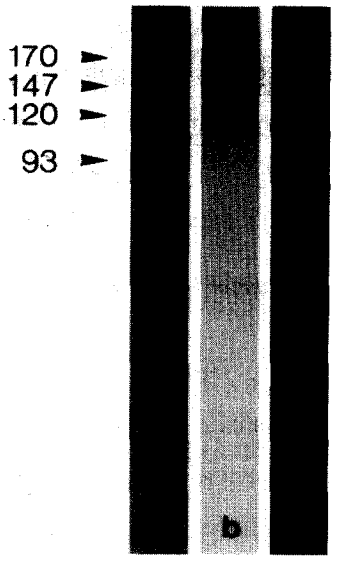

Fig.1. SDS-PAGE of purified *B. stearothermophilus* NRS 2004/3a S-layer glycoprotein (a) stained with Coomassie blue, (b) after periodic acid-Schiff staining, and (c) after HF treatment and protein staining. The apparent molecular masses of the sample components are indicated (in kDa).

3. RESULTS

3.1. *Characterization of GP I*

GP I contains only rhamnose and no contamination originating from GP II (detectable as di-*N*-acetylmannuronic acid) [6]. The composition of this material is given in table 1.

Table 1

Amino acid and monosaccharide composition of GP I and the linkage component

Component	Glycopeptide I		Asparaginyl-rhamnose
	(%)[a]	(molar ratio)	(molar ratio)
Amino acids			
Total	3.5		
Asx		2.8	1.0
Thr		1.0	–
Ser		2.0	–
Ala		1.2	–
Carbohydrates			
Rha	92	~150[b]	1.2

[a] Based on dry weight

[b] Based on an apparent molecular mass of ~20 kDa on a calibrated Bio-Gel P-30 column

3.2. *SDS-PAGE of the S-layer glycoprotein after HF treatment*

As reported [7], slab gel electrophoresis of the intact S-layer glycoprotein revealed 4 bands of apparent molecular masses 93, 120, 147 and 170 kDa (fig.1, lane a). The three higher bands stained with the periodic acid-Schiff reagent, indicating their content of carbohydrate (fig.1, lane b). The ratio of glycosylated to non-glycosylated protein (93 kDa) was estimated by preparative SDS-PAGE as ~1:5 (not shown).

After treatment with HF and dialysis the content of the dialysis bag appeared turbid, indicating undisintegrated S-layer material. SDS-PAGE of an aliquot of this suspension showed the presence of only one high-molecular-mass band (93 kDa) accompanied by products of lower molecular mass (fig.1, lane c). The periodic acid-Schiff-positive bands had disappeared completely. Amino acid analysis of the residual protein revealed no differences in composition compared to intact S-layer glycoprotein [7].

3.3. *HF treatment of GP I*

After hydrolysis of GP I with HF at 0°C, low-

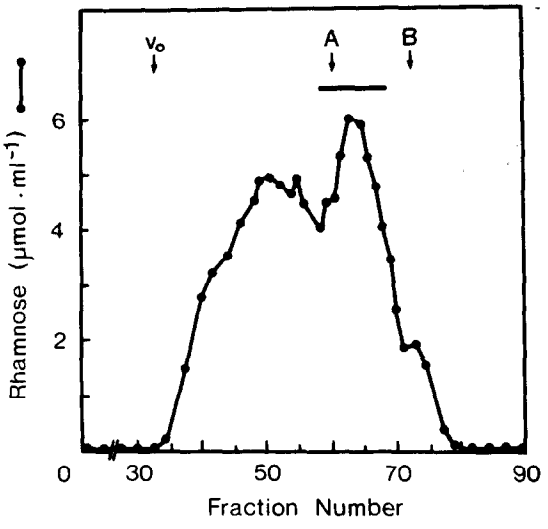

Fig.2. Separation of the products after HF treatment of GP I on a Bio-Gel P-2 column (1 × 113 cm). Fractions (1 ml) were collected and pooled as indicated by the bar. v_o, A, and B indicate the void volume, a disaccharide (A) and a monosaccharide (B).

molecular-mass reaction products were separated on a calibrated Bio-Gel P-2 column (fig.2). A mixture of degradation products was eluted within the void volume. TLC analysis of the fractions eluted in the position of a disaccharide standard showed essentially a single component migrating between rhamnose and aspartic acid. This material gave positive reactions with both the orcinol and ninhydrin spray reagents. The collected fractions indicated in fig.2 were subjected to ion-exchange chromatography on DEAE Bio-Gel A for further purification. Only one peak was eluted from the column at a salt concentration of 0.22 M ammonium bicarbonate. This material did not contain any free rhamnose (not shown). Upon rechromatography on the Bio-Gel P-2 column it gave a single peak with a molecular mass of ~300 Da (not shown). Hydrolysis and amino acid analysis showed aspartic acid to be the only amino acid (fig.3a). GC analysis of the borohydride-reduced, peracetylated hydrolysate revealed rhamnitol to be the only carbohydrate constituent (fig.3b). By colorimetry, a molar ratio of aspartic acid to rhamnose of 1.0:1.2 was estimated in the hydrolysate.

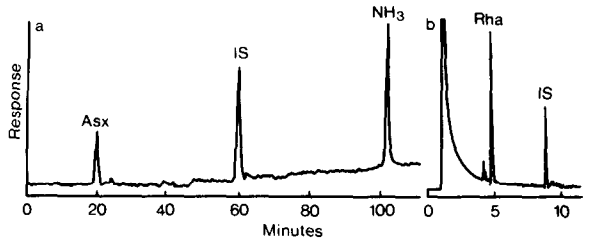

Fig.3. Analysis of the carbohydrate-protein linkage of GP I. After hydrolysis with 4 M trifluoroacetic acid (a) on the amino acid analyzer; IS, internal standard (norleucine); and (b) by GC; IS, internal standard (*myo*-inositol).

4. DISCUSSION

For the structural elucidation of the protein-carbohydrate linkage of GP I of *B. stearothermophilus* NRS 2004/3a [5], this glycopeptide (table 1) has been solvolyzed by HF (a reagent which cleaves *O*-glycosidic linkages, but leaves *N*-glycosidic ones and peptides intact [13]) to isolate an aminoacyl saccharide. Despite extensive pronase digestion of the intact S-layer glycoprotein according to Wieland et al. [14] we were unable to isolate an asparagine saccharide, presumably due to steric hindrance of the protease by the intact glycan chain [5]. On the other hand, SDS-PAGE of the deglycosylated glycoprotein has revealed a certain degradation of the protein which might have been induced by traces of water in the HF [14]. Because an identical HF hydrolysis procedure has been applied to GP I, a similar unspecific cleavage is expected, which could yield the protein-carbohydrate linkage region among other components in the reaction mixture. Characterization of the low-molecular-mass fraction obtained after ion-exchange and gel-permeation chromatography confirmed this assumption. The glycan chain of GP I of *B. stearothermophilus* NRS 2004/3a [5] is linked to the protein via a novel *N*-glycosidic linkage between rhamnose and asparagine. It is unknown which of the rhamnoses of the trisaccharide repeating unit [5] is linked to the amino acid residue, or whether the rhamnose is part of a 'core' saccharide. The observation of aspartic acid, serine, threonine and alanine in the peptide portion of GP I (table 1) before HF treatment is consistent with the presence of the established acceptor sequence Asn-X-Thr(Ser) [15] found so far in all *N*-glycosidic linkages described; i.e. Asn-GlcNAc [15], Asn-Glc [14], and Asn-GalNAc [16].

The existence of glycoproteins in prokaryotes has been believed to be restricted to archaebacteria [17–20], although in the past there were several reports on glycoproteins in prokaryotic cells (e.g. [21,22]). However, substantiating evidence of such claims is still lacking. Only in a few examples such as the endogenous, autolytic *N*-acetylmuramoyl-hydrolase of *Streptococcus faecium* [23], the cell surface glycoprotein of *Myxococcus xanthus* [24], or the glycoprotein toxin crystals of *B. thuringiensis* ssp. *israelensis* [25] has strong evidence for eubacterial glycoproteins been provided. Here, we demonstrate by isolation of the protein-carbohydrate linkage region of the glycoprotein for the first time that eubacterial species possess the ability to glycosylate proteins.

Acknowledgements: We thank Dr F.M. Unger for critical reading of the manuscript, Martina Nelson for her help with the GC analyses and T. Dalik for the amino acid analyses. This work was supported by the Österreichisches Bundesministerium für Wissenschaft und Forschung.

REFERENCES

[1] Sleytr, U.B. and Messner, P. (1983) Annu. Rev. Microbiol. 37, 311–339.
[2] Sleytr, U.B. and Thorne, K.J.I. (1976) J. Bacteriol. 126, 377–383.
[3] Sleytr, U.B., Sára, M., Küpcü, Z. and Messner, P. (1986) Arch. Microbiol. 146, 19–24.
[4] Messner, P. and Sleytr, U.B. (1988) in: Crystalline Bacterial Cell Surface Layers (Sleytr, U.B. et al. eds) Springer, Berlin, in press.
[5] Christian, R., Schulz, G., Unger, F.M., Messner, P., Küpcü, Z. and Sleytr, U.B. (1986) Carbohydr. Res. 150, 265–272.
[6] Messner, P., Sleytr, U.B., Christian, R., Schulz, G. and Unger, F.M. (1987) Carbohydr. Res. 168, 211–218.
[7] Küpcü, Z., März, L., Messner, P. and Sleytr, U.B. (1984) FEBS Lett. 173, 185–190.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[9] Keleti, G. and Lederer, W.H. (1974) Handbook of Micromethods for the Biological Sciences, p.47, Van Nostrand-Reinhold, New York.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Messner, P., Hollaus, F. and Sleytr, U.B. (1984) Int. J. Syst. Bacteriol. 34, 202–210.
[12] Messner, P., Pum, D. and Sleytr, U.B. (1987) J. Ultrastruct. Mol. Struct. Res., in press.
[13] Mort, A.J. and Lamport, D.T.A. (1977) Anal. Biochem. 82, 289–309.
[14] Wieland, F., Heizer, R. and Schaefer, W. (1983) Proc. Natl. Acad. Sci. USA 80, 5470–5474.
[15] Kornfeld, R. and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664.
[16] Paul, G., Lottspeich, F. and Wieland, F. (1986) J. Biol. Chem. 261, 1020–1024.
[17] Mescher, M.F. and Strominger, J.L. (1976) J. Biol. Chem. 251, 2005–2014.
[18] Mescher, M.F. (1981) Trends Biochem. Sci. 6, 97–99.
[19] Kandler, O. (1982) Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg., I. Abt. Orig. C3, 149–160.
[20] Sharon, N. (1987) J. Biol. Chem. 262, 13–18.
[21] Okuda, S. and Weinbaum, G. (1968) Biochemistry 7, 2819–2825.
[22] Abe, M. and Kimoto, M. (1984) Microbiol. Immunol. 28, 841–846.
[23] Kawamura, T. and Shockman, G.D. (1983) J. Biol. Chem. 258, 9514–9521.
[24] Maeba, P.Y. (1986) J. Bacteriol. 166, 644–650.
[25] Muthukumar, G. and Nickerson, K.W. (1987) Appl. Environ. Microbiol. 53, 2650–2655.