^{31}P NUCLEAR MAGNETIC RESONANCE STUDIES ON THE PHOSPHOGLYCOPEPTIDES OBTAINED FROM RAT BRAIN GLYCOPROTEIN

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Received 4 March 1976

1. Introduction

^{31}P Nuclear magnetic resonance (^{31}P n.m.r.) studies have provided information on serum lipoproteins [1], red blood cells [2] and other biological systems [3,4]. Recently, structural elucidation of the phosphates present in the phosphomannans isolated from yeast have been reported by Costello et al. [5]. Presently, ^{31}P n.m.r. has been applied to a previously unreported glycopeptide-phosphate obtained from rat brain glycoproteins [6].

2. Materials and methods

The total glycopeptides from 90 whole rat brains were prepared according to the procedure of Javaid et al. [7]. Affinity chromatography on Con A-Sepharose 4B was utilized to separate a glycopeptide fraction that bound to the lectin (Con A-positive) from glycopeptides that do not bind (Con A-negative). After elution of the Con A-positive glycopeptide fraction with 2% α-methylmannoside, the two fractions were desalted by ultrafiltration using UM 2 membranes and gel filtration on Bio-gel P-2 before being studied under the magnetic resonance conditions previously reported useful for a study of the yeast phosphomannans [5]. Hexose was determined by the method of Dubois et al. [8], glucosamine by the method of Boas [9] and individual sugars were identified and quantitated by the g.l.c. method of Porter [10]. A Bruker HFX-5 spectrophotometer [11,12] operating at 36.43 MHz for ^{31}P (^{1}H field equivalent to 90 MHz) was used and equipped for the processing [2,13] of ^{31}P Fourier transformed spectra with a 16K Nicolet computor. Deuterium field frequency stabilization was employed using a ^{2}H signal derived from 10% D_2O added to the sample. The chemical shifts were obtained relative to 85% orthophosphoric acid [4,14] as the zero ppm reference.

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3. Results and discussion

Con A-negative glycopeptides, which contain approx. 70% of the total rat brain glycopeptide-hexose [7], gave no detectable phosphorus signal with up to 20 K accumulations. However, the Con A-positive glycopeptides which account for about 30% of the total brain glycoprotein-hexose [7] provided a sharp phosphorus signal within 2 K accumulations. Exhaustive Diaflo (Amicon, Lexington, Mass.) ultrafiltration using a UM 2 membrane, desalting by collecting the void volume of gel filtration on Bio-Gel P-2 (5 X 30 cm columns), and repeated extraction with chloroform–methanol (2:1 and 1:2, v/v) failed to diminish or alter the signal. This glycopeptide preparation consisted predominately of mannose and *N*-acetylglucosamine (Man/GlcNAc = 3.1). Small amounts of fucose and galactose were present (Gal/GlcNAc and Fuc/GlcNAc = 0.2). This biological

Table 1
^{31}P N.m.r. analysis of standards and phosphoglycoprotein material from brain

Substance		pH[a]	Coupling J (Hz)	Chemical shift Hz	ppm
(1)	L-phosphoserine	4.22	–	–	–
		11.22	–	−140.6	−3.81
(2)	D-mannose-6-phosphate[b]	4.22	5.9	−27.4	−0.75
		11.24	6.1	−158.1	−4.34
(3)	α-D-glucosyl-phosphate[b]	4.27	7.8	48.1	1.32
		11.20	7.78	−81.3	−2.23
(4)	α-D-galactosyl-phosphate[b]	4.27	6.6	47.0	1.29
		11.28	7.3	−82.8	−2.27
(5)	Con A-binding phospho-glycopeptide from brain	4.22	–	−33.1	−0.90
		11.20	4.28	−164.5	−4.51
(6)	Phosphohexose obtained from [5] after acid hydrolysis	4.23	–	−1.2	−0.03
		11.24	–	−119.6	−3.28

[a] Titration was performed on the free acid with tert-butylammonium hydroxide.
[b] Data previously reported by Costello et al. [5].

glycopeptide-phosphate preparation was further studied and compared with model compounds.

The chemical shifts of the Con A-binding brain phosphoglycopeptides, the phosphohexose residue obtained from these glycopeptides by acid hydrolysis, and several standard phosphate compounds were compared (table 1). The data are reported for these compounds after titration of the free acid to the specified pHs of 4.22 and 11.20 [5,15] with tertiary-butyl ammonium hydroxide. Proton coupling constants obtained when coupled spectra were performed as described previously [5] are included in the table. Data pertaining to some of the standard compounds has been reported previously by Costello et al. [5]. The change in the chemical shifts observed at the two pH conditions utilized revealed that the phosphate groups in the phosphoglycopeptides were bound by means of monoester phosphate linkages [5]. Of further interest was the finding that the coupled spectra under conditions for proton coupling was a doublet (fig.1). This observation indicates that the monoester phosphate in the glycopeptide is attached to a methine carbon. The demonstration that a phosphohexose can be obtained from the phosphoglycopeptide preparation by acid hydrolysis [7], and the demonstration by ^{31}P n.m.r. of a monoester phosphate attached to a methine

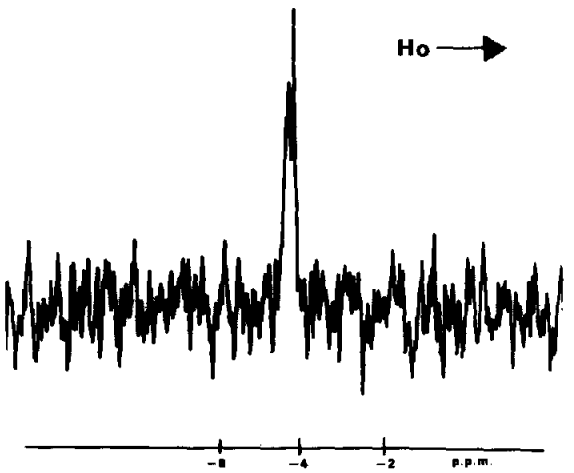

Fig.1. ^{31}P n.m.r. coupled spectra obtained from Con A-binding rat brain glycopeptides. Chemical shifts are relative to 85% orthophorphoric acid as the zero (0) ppm reference. The spectra were obtained through the use of Fourier transform n.m.r. techniques: spectra width employed, 500 Hz; acquisition time 1 sec (dwell time 1000 μsec per point, for 8192 data points); cyclic time, 9 sec. At least 5 h acquisition time was needed to obtain 2 K accumulations. Sample volume was 3.0 ml contained in a 10 mm spinning tube. About 2 K accumulations were averaged per spectrum, temperature 28°C. Deuterium field stabilization was employed through the use of resonance from 10% D_2O added to the sample.

carbon suggests a linkage of phosphate with either the C1, C2, C3 or C4 carbon positions in the hexose. In rat brain glycopeptides, all of the anomeric carbons are involved in glycosidic linkages and reducing sugars are absent [16]. Thus, it appears that the monoester phosphate is attached to the hydroxyl group of one of the methine carbons located in either the C2, C3, or C4 positions. It is possible that the phosphate is in the anomeric position and the sugar is non-reducing because of this location, but this possibility is ruled out by ^{31}P n.m.r. studies of the free phosphohexose compared to the model compounds (table 1).

^{31}P N.m.r. analysis of the isolated phospho-hexose obtained by ion exchange chromatography after acid hydrolysis of the Con A-binding acidic glycopeptides [7] again demonstrated the phosphate to be in a monoester linkage as shown by the chemical shift changes at the pHs studied. In addition, the chemical shift obtained, −3.28 ppm, when titrated to pH 11.20 (table 1) indicated that the properties of the phosphohexose did not correspond to any of the 1- or 6-phosphohexose standards available, and the properties of the bound phosphate differed from that of phosphoserine.

The different chemical shifts obtained for the phosphorus present in the more complex phosphoglycopeptide (−4.51 ppm at pH 11.20) compared to the simpler phosphohexose structure (−3.28 ppm under the same conditions) provides additional evidence for the important effect macromolecular conformation plays on chemical shift [5]. The further downfield shift of the phosphate group when present in the glycopeptide is not fully understood, but presumably is the result of more deshielding when the phosphate interacts with the increased number of molecules available in the glycopeptide rather than just its nearest neighbors as would be the case in the hexose alone. In the latter case, the chemical shift of the phosphate in the hexose would be affected by the stereochemical configuration of the nearby hydroxyl groups alone. With the additional information that the phosphate is linked to a methine carbon, it should be possible to predict the position to which it is linked. However, the exact position cannot be determined as yet since the necessary model compounds are not available nor is the precise identification of the hexose established. Although present data indicate the hexose to be mannose, this identity remains to be confirmed.

Proton magnetic resonance studies of the phosphohexose recovered from the phosphoglycopeptide confirmed that the hexose was not α-D-mannose 1-phosphate nor D-mannose-6-phosphate. Although the spectra obtained were consistent with D-mannose the differences observed could not be resolved due to limited sample, nor fully interpreted because of the nearby HDO peak. The similarity of the proton n.m.r. spectra of the phosphohexose to these standard mannose compounds did lend further support to the notion that the phosphohexose is phosphomannose.

The ^{31}P n.m.r. studies reported here have provided structural information on the phosphoglycopeptide and phosphohexose isolated from phosphoglycopeptides obtained from rat brain glycoproteins. The phosphate was found to be in a monoester linkage to the hexose, and to be attached to a methine carbon. In addition, the further downshift observed for the glycopeptide phosphate when compared to the hexose-phosphate derived from the glycopeptide supports the suggestion [5] that the phosphate groups associated with macromolecules in solution interact markedly with their conformational tertiary molecules and not just the adjacent configurational hydroxyls.

Acknowledgements

We wish to thank Dr Glonek for helpful comments during the ^{31}P n.m.r. studies and Dr Venton for his assistance on the proton n.m.r. studies. We thank the Research Resources Center at the University of Illinois for making their facilities available to us. This research was supported in part by a grant from the National Science Foundation (Grant No. 33624) and from the American Heart Association (Grant 74-1011). L.G. Davis was supported by a traineeship provided by a USPHS (USPMH) award to the University of Illinois, School of Medicine, Chicago, Illinois. This research is part of the dissertation of L.G. Davis to be submitted to the Univ. of Illinois in partial fulfillment for the requirements of the PhD degree.

References

[1] Henderson, T. O., Kruski, A. W., Davis, L. G., Glonek, T. and Scanu, A. M. (1975) Biochemistry 14, 1915–1920.

[2] Henderson, T. O., Costello, A. J. R. and Omachi, A. (1974) Proc. Natl. Acad. Sci., USA 71, 2487–2490.
[3] Glonek, T., Kleps, R. A. and Myers, T. C. (1974) Science 185, 352–355.
[4] Glonek, T., Henderson, T. O., Hilderbrand, R. L. and Myers, T. C. (1974) Science 169, 192–194.
[5] Costello, A. J. R., Glonek, T., Slodki, M. E. and Seymour, F. (1975) Carbohyd. Res. 42, 23–37.
[6] Davis, L. G., Javaid, J. I. and Brunngraber, E. G. (1976) FEBS Lett., previous paper.
[7] Javaid, J. I., Hof, H. and Brunngraber, E. G. (1975) Biochim. Biophys. Acta 404, 74–82.
[8] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[9] Boas, N. F. (1953) J. biol. Chem. 234, 553–563.
[10] Porter, W. H. (1975) Anal. Biochem. 63, 27–43.
[11] Glonek, T., Lunde, M., Mudgett, M. and Myers, T. C. (1971) Arch. Biochem. Biophys. 142, 508–513.
[12] Henderson, T. O., Glonek, T., Hilderbrand, R. L. and Myers, T. C. (1972) Arch. Biochem. Biophys. 149, 484–497.
[13] Glonek, T., Henderson, T. O., Kruski, A. W. and Scanu, A. M. (1974) Biochim. Biophys. Acta 348, 155–161.
[14] Crutchfield, M. M., Dungan, C. H., Letcher, J. H., Mark, V. and Van Wazer, J. R., (1967) in: Topics in Phosphorus Chemistry (Grayson, M. and Griffith, E. J., eds.) Vol 5, Interscience, New York.
[15] Glonek, T., Kleps, R. A., Griffith, E. J. and Myers, T. C. (1975) Phosphorus 5, 157–164.
[16] Brunngraber, E. G. (1972) Adv. Exptl. Med. and Biol. 25, 17–49.