ELSEVIER

Carbohydrate Research 306 (1998) 559–562

CARBOHYDRATE RESEARCH

Note

Identification of phosphate substitution sites by NMR spectroscopy in a water-soluble phosphorylated (1→3)-β-D-glucan

Douglas Lowman [a], Harry Ensley [b], David Williams [cd]*

[a] *Research Laboratories, Eastman Chemical Company, Kingsport, TN 37662-5150, USA*

[b] *Department of Chemistry, Tulane University, New Orleans, LA 70115, USA*

[c] *Department of Surgery and* [d] *Immunopharmacology Research Group, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614-0575, USA*

Received 14 November 1997; accepted 29 December 1997

Abstract

Detailed analysis of the site-specific phosphorylation of a glucan phosphate from *Saccharomyces cerevesiae* has been carried out by 2D NMR techniques. Phosphorylation has been shown to be limited to the C-6 and C-2 positions, with the C-6 resonance showing two slightly different environments. Phosphorylation at C-4 is hindered due to proximity effects with the neighboring glucose ring oxygen. Noncovalently bound, nondialyzable phosphate appears to be coordinated to the nonphosphorylated HO-2 group of the helical polymer. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Site-specific phosphorylation; Glucan phosphate; Noncovalently bound, Nondialyzable phosphate

Naturally occurring (1→3)-β-linked glucans belong to the class of drugs known as biological response modifiers (BRMs) [1]. Numerous studies have demonstrated that (1→3)-β-D-glucans exhibit considerable immunomodulatory activity [1,2] following binding of the glucan ligand to its cognate receptor on immune competent cells [3]. We have studied (1→3)-β-D-glucans isolated from the inner cell wall of *Saccharomyces cerevisiae* [4,5]. Upon initial isolation from *S. cerevisiae*, the (1→3)-β-D-glucan is a water-insoluble microparticulate [4,5]. The microparticulate glucan is employed as the starting material for the production of water-soluble pharmaceutical grade (1→3)-β-D-glucans [4,5].

We have extensively studied the immunobiological properties of one of these water-soluble glucans, glucan phosphate [1–3]. We have also focused efforts on understanding the chemistry of (1→3)-β-D-glucan phosphate in order to associate structural determinants with biological activity [4–6]. Muller et al. [6] have reported a Mark–Houwink (α) value of 0.354 for glucan phosphate, suggesting an aqueous solution conformation of a partially solvated

* Corresponding author. Fax: 001 615 929 6459.

PII S0008-6215(98)00014-7

or perturbed coil. The aqueous conformation of glucan phosphate may be influenced by the presence of the phosphate groups. The present NMR study was undertaken to determine phosphate group substitution sites along the glucan phosphate polymer backbone.

The ^{31}P NMR spectrum of the glucan phosphate is shown in Fig. 1. In addition to inorganic phosphate at 0 ppm, there is evidence for phosphate groups in several different magnetic environments. In this study, we employed phase-sensitive double-quantum-filtered homonuclear correlation spectroscopy (COSY) [7], inverse-detected ^{1}H–^{31}P heteronuclear correlation spectroscopy (HPCOSY) [8,9] and phase-sensitive total correlation spectroscopy (TOCSY) [10] to assign specific ^{31}P NMR resonances to those phosphate groups attached to the glucan and to identify specific substitution positions for the phosphate groups on the glucan.

Figure 2 shows the COSY spectrum and Fig. 3 shows the HPCOSY spectrum for the glucan phosphate. The HPCOSY spectrum shows three correlations. By correlating the HPCOSY crosspeaks to correlations in the COSY spectrum, site-specific assignments for the phosphate groups can be made.

The most deshielded HPCOSY crosspeak correlates the phosphate resonance at 1.34 ppm with a proton resonating at 3.97 ppm. The HPCOSY crosspeak with intermediate shielding is more diffuse, correlating a broad phosphate resonance in the region of 0.8–1.0 ppm to a proton resonating at 3.95 ppm. The broad phosphate resonance in the region of 0.8–1.0 ppm and diffuse crosspeak for this resonance suggest similar—but more than one—environments for the phosphate group assigned to this resonance. These resonances near 3.96 ppm are part of the coupling pattern resulting from germinal protons of C-6 methylene groups based on the COSY spectrum. The correlation to the methylene protons at C-6 indicates that the phosphate group is attached to C-6. Assignments of phosphate attached to C-6 are consistent with data reported previously for D-glucose-6-phosphate [11]. The remaining crosspeak in the HPCOSY spectrum correlates the phosphate resonance at 0.33 ppm to a proton resonance at 3.89 ppm. This proton is part of a coupled network containing protons that resonate at 4.49 and 3.64 ppm. These resonances are assigned to a three-spin network that is part of a glucose residue containing a phosphate group. ^{1}H NMR chemical shift

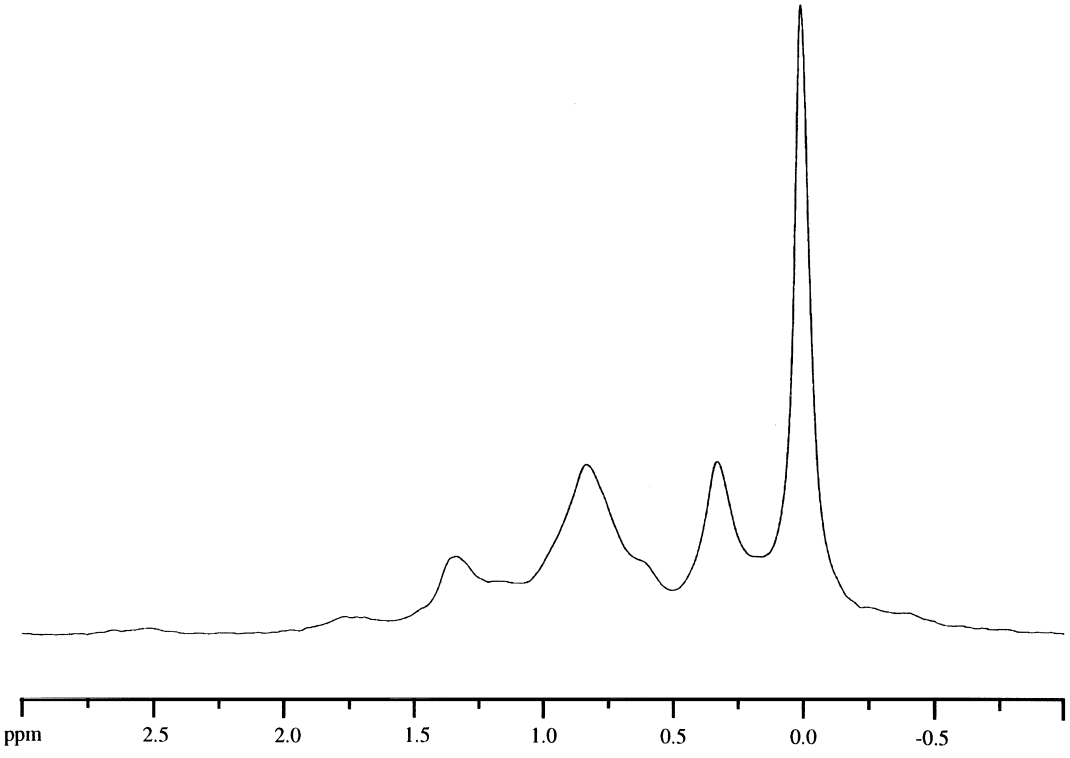

Fig. 1. 1D ^{31}P NMR spectrum of (1→3)-β-D-glucan phosphate showing phosphate groups in several different magnetic environments as well as inorganic phosphate.

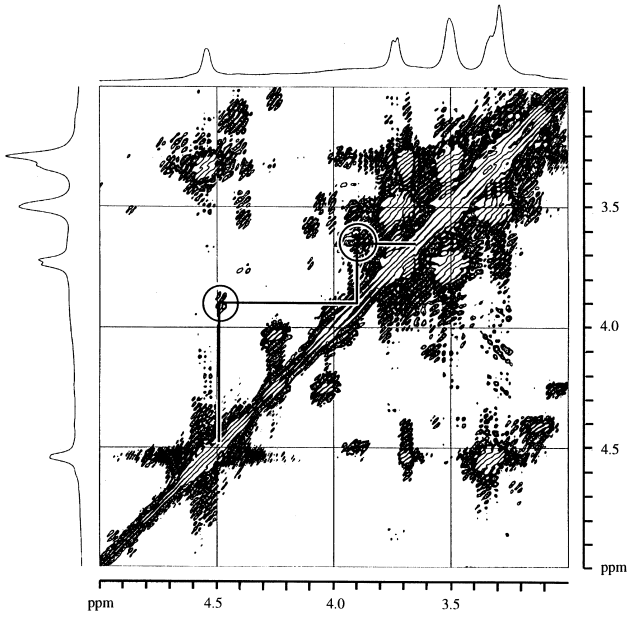

Fig. 2. An expanded COSY 2D ^{1}H NMR spectrum of (1→3)-β-D-glucan phosphate

assignments for the glucose residues in the nonphosphorylated glucan have been reported [5]. The resonance at 4.49 ppm, coupled to the resonance at 3.89 ppm, is assigned to H-1 of the glucose phosphate residue due to its close proximity to the chemical shift of H-1 (4.52 ppm) from the nonphosphorylated glucose residue. The resonance at 3.89 ppm is thus assigned to H-2, which indicates that the phosphate group is attached to C-2. This assignment of H-2 is confirmed by its correlation to the resonance at 3.64 ppm that is assigned to H-3 due to its close proximity to the resonance of H-3 (3.46 ppm) in the nonphosphorylated glucan. These assignments were confirmed by a TOCSY experiment.

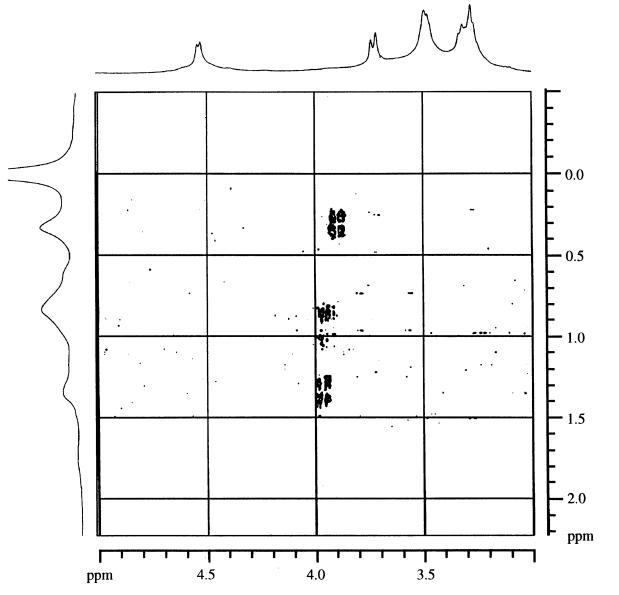

Fig. 3. Inverse-detected ^{1}H–^{31}P correlated 2D NMR spectrum of (1→3)-β-D-glucan phosphate showing three correlations.

All three correlations in the HPCOSY spectrum are assigned to specific sites on a glucose residue. Clearly the remaining ^{31}P NMR resonances in Fig. 1 are not associated with this glucan phosphate.

There is no evidence in our data for a measurable presence of a phosphate group attached to C-4. If this glucan exists in a helical solution conformation, the hydroxyl group attached to C-4 is sufficiently close to the oxygen in the neighboring glucose ring to encourage hydrogen bonding, thus inhibiting phosphorylation at C-4.

Williams et al. have reported that the total phosphate concentration in the glucan to be approximately 2.23% [4]. Our data indicate that most of the available phosphate, present as inorganic phosphate or phosphoric acid, is not covalently bound to the polymer. Attempts to remove these noncovalently bound phosphate groups by extensive dialysis have proven unsuccessful [4]. We speculate that the noncovalently bound phosphate groups are strongly coordinated to the many nonphosphorylated C-2 hydroxyl groups within the center of the helix. Thus, the noncovalently bound phosphate is retained by the glucan. Based on elemental analysis we had previously reported a phosphate substitution every seventh glucose subunit along the polymer backbone [4]. The present data indicate that the degree of phosphate substitution is substantially lower than previously thought. This discrepancy is due to the presence of the nondialyzable, noncovalently linked phosphate at the interior of the polymer helix.

In conclusion, COSY, HPCOSY and TOCSY experiments have been combined to provide a detailed analysis of the site-specific phosphorylation of a glucan phosphate from *S. cerevisiae*. Phosphorylation occurs to a limited extent at the C-6 and C-2 positions of the glucose ring. Evidence for phosphorylation in two slightly different magnetic environments for C-6 is presented. Phosphorylation at C-4 is hindered due to proximity effects with the neighboring glucose ring oxygen possibly resulting in hydrogen bond formation. Noncovalently bound phosphate, which cannot be dialyzed from the polymer, appears to be strongly

coordinated to the nonphosphorylated C-2 hydroxyl groups inside the helical polymer.

1. Experimental

Glucan phosphate.—Glucan phosphate was prepared as previously described by our laboratory [4]. Briefly, microparticulate glucan [4] was dissolved in dimethyl sulfoxide and heated in the presence of urea and phosphoric acid. The resulting water-soluble product was dialyzed to remove unreacted reagents and then lyophilized. The physicochemical characteristics were established as previously described [4–6].

NMR spectroscopy.—The glucan phosphate was dissolved in Me_2SO-d_6 at 80 °C. All spectra were obtained using 5-mm OD NMR tubes at 80 °C with a Bruker DXR-500 NMR spectrometer. The glucan phosphate (70 mg) was dissolved in 1 mL of solvent. The COSY spectrum was acquired in a 512×256 matrix using 400 scans per row, linear back predicted, and zero-filled to a 1024×1024 matrix. Spectral width was 4.5 ppm centred at 3.7 ppm. The HPCOSY spectrum was acquired in a 1024×256 matrix using 350 scans per row, linear back predicted, and zero-filled to a 1024×1024 matrix. Sweep width in F2 was 4.5 ppm centred at 3.7 ppm and in Fl was 3.0 ppm centred at 0.75 ppm.

References

[1] D.L. Williams, A. Mueller, and W. Browder, *J. Endotoxin Res*., 2 (1995) 203–208.
[2] D.L. Williams, A. Mueller, and W. Browder, *Clin. Immunother*., 5 (1996) 392–399.
[3] A. Mueller, P. Rice, H. Ensley, P. Coogan, J.H. Kalbfleisch, J. Kelley, E.J. Love, C.A. Portera, T. Ha, W. Browder, and D.L. Williams, *J. Immunol*., 156 (1996) 3418–3425.
[4] D.L. Williams, R.B. McNamee, E.L. Jones, H.A. Pretus, H.E. Ensley, I.W. Browder, and N.R. DiLuzio, *Carbohydr. Res*., 219 (1991) 203–213.
[5] H.E. Ensley, B. Tobias, H.A. Pretus, R.B. McNamee, E.L. Jones, I.W. Browder, and D.L. Williams, *Carbohydr. Res*., 258 (1994) 307–311.
[6] A. Muller, H.A. Pretus, D.L. Williams, R.B. McNamee, E.L. Jones, and I.W. Browder, *J. Chromatogr*., 66 (1995) 283–290.
[7] A. Derome and M. Williamson, *J. Magn. Reson*., 88 (1990) 177–185.
[8] A. A. Maudsley and R.R. Ernst, *Chem. Phys. Lett*., 50 (1977) 368–372.
[9] V. Sklenar, H. Miyashiro, G. Zon, H.T. Miles, and A. Bax, *FEBS Letters*, 208 (1986) 94–98.
[10] A. Bax and D.G. Davis, *J. Magn. Reson*., 65 (1985) 355–360.
[11] P.M.L. Robitaille, P.A. Robitaille, G.G. Brown, Jr., and G.G. Brown, *J. Magn. Reson*., 92 (1991) 73–84.