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Galactofuranosyl-containing glycopeptide of *Penicillium charlesii*

Vacuum ultraviolet circular dichroism

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The far and vacuum u.v. circular dichroism (CD) of peptidophosphogalactomannan from *P. charlesii* is reported to 182.5 nm in aqueous and aqueous/organic solvents, and to 150 nm in films. CD of films of the peptide-free derivative is reported to 150 nm. On the basis of these data we conclude that the peptide chain is unordered, and may best be described as a hydrated coil showing some stiffness. The small observed saccharide CD may result from cancellation of contributions from the various saccharide structures present or from a lack of repeating secondary structure.

Key words: circular dichroism; peptidophosphogalactomannan

Fungal glycoproteins and glycopeptides have been found in or associated with cell walls and in the extracellular environment. Fungi secrete degradative glycoenzymes which function in the organism's nutrition (1).

Current ideas concerning the structure and function of glycoproteins have been reviewed (2). Glycoproteins are glycosylated at either asparaginyl residue(s) forming an *N*-glycosyl linkage or they are glycosylated at seryl and/or threonyl residue(s). Some glycoproteins contain both *N*- and *O*-glycosyl linkages to the proteins. In both cases, sites of glycosylation have been correlated with β-turns (3, 4). It has been hypothesized that glycosylation may function in intracellular recognition and transport (5) and in protection from proteases (4) and denaturation. Tunicamycin, which blocks glycosylation of asparaginyl residues, does not always block secretion of the protein portion of glycoproteins; in some instances degradation of the saccharide-deficient compound occurs. Thus a role for glycosylation in intracellular transport is not universal and requires further elaboration; the hypothesis for a protective role is supported (see review by Montreuil (2)). Glycoproteins have roles in molecular and intercellular recognition and aggregation, in control of membrane permeability, and as glycoenzymes (2). They are also found in fungal cell walls (1).

Peptidophosphogalactomannan (pPGM)* has a molecular weight of 70,000, contains galactose in its furanosyl form, and is found in the growth medium of *Penicillium charlesii*. Other compounds of similar composition containing gal-

Abbreviations used: pPGM, peptidophosphogalactomannan; PGM, phosphogalactomannan; CD, circular dichroism; u.v., ultraviolet; VUCD, vacuum ultraviolet circular dichroism; BSA, bovine serum albumin; TFE, 2,2,2-trifluoroethanol.

actofuranosyl residues are found in various fungi (1). pPGM contains: a mannan backbone of about 100 residues with α–1→2 and α–1→6 linkages in a three-to-one ratio (6, 7); about 10 branches each averaging 25 β–1→5 linked galactofuranosyl residues which are linked to C–3 of a C–2 substituted mannosyl residue of the backbone (6, 7); about 10 phosphodiester residues, each bound to a mannosyl residue of the backbone at C–6 and either ethanolamine, choline, or possibly other *N*-methylated derivatives of ethanolamine (8–11); and a heterogeneous polypeptide containing about 30 amino acyl residues, half of which are seryl and threonyl residues. About three-fourths of the β-hydroxyl-containing amino acyl residues are linked *O*-glycosidically, one to the mannan backbone and others to mannosyl, mannobiosyl, and mannotriosyl residues (6, 8). The peptide contains only traces of aromatic and sulfur-containing residues (8, Tonn & Gander, unpublished observation). Gander & Laybourn (11) have hypothesized that pPGM is derived from various glycoproteins and that it is involved in recognition and transport of glycoproteins and in survival under stress of nutrient depletion.

CD in the far u.v. region has been used to estimate secondary structure in proteins and in the protein portion of glycoproteins (12–15); Brahms & Brahms (16) have extended the method to include CD in the vacuum u.v. region. Also, Bush *et al.* (17) have subtracted spectra of model saccharides extended to the vacuum u.v. from that of fish antifreeze glycoprotein to ascertain secondary structure, a procedure which has been investigated and used in the far u.v. for a number of glycoproteins and glycopeptide derivatives (e.g. 18–20).

Extension of CD to the vacuum region has allowed direct investigation of transitions in non-substituted saccharides (21, 22). Nelson & Johnson (22–24) have studied series of monosaccharides and methylglycosides, and conformational information about polysaccharides has been derived (e.g. 25). However, no unified theoretical or empirical method for discussing VUCD and polysaccharide conformation has been developed. Investigation of the far and vacuum u.v. CD of pPGM was undertaken in an attempt to derive conformational information about the molecule and as part of a continuing study of polysaccharide VUCD.

EXPERIMENTAL PROCEDURES

P. charlesii was cultured and pPGM isolated as described by Gander *et al.* (6). pPGM was treated with 0.4 N NaOH, which cleaves *O*-glycosidic bonds to seryl and threonyl residues, and PGM, the high molecular weight derivative, was isolated by gel filtration (6). This procedure was repeated to ensure formation of peptide-free PGM. Choline, which is linked to the galactomannan through phosphodiester bonds, is also cleaved by this treatment (10).

Protein was determined by a modifed Lowry assay (26) using BSA as a standard.

Total saccharide was determined by the phenol-sulfuric acid method (27) using galactose and mannose standards and the galactose: mannose ratio of 265:120 for pPGM and 265: 100 for PGM. The milligram equivalents found were multiplied by 162/180 to correct hexosyl molecular weights of the references to anhydrohexosyl molecular weights of the polymers.

Solutions were made by dissolving weighed amounts of lyophilized samples of pPGM and PGM in distilled deionized water or in a TFE/water solvent. Solutions made to saturating concentrations were centrifuged to remove particulate matter. The pH of aqueous solutions were 6.4. Films were cast on CaF_2 disks, 1.9 cm in diameter, by evaporating solutions over drierite and NaOH.

The instrumentation used in recording the VUCD spectra has been described (21). Spectra were recorded with a bandwidth of 3.2 nm at 0.2 nm/min and 1 nm/min with time constants of 100 and 30 s, respectively; the temperature was approximately 18°. Solution spectra were obtained in 24–504 micron path length fused silica cells. Films were checked for birefringence by a 90° rotation about the optical axis. Ellipticities were determined by comparison to a D-camphor-10-sulfonic acid standard which has a maximum at 290 nm with $[\theta]_{290} = 7780$ deg · cm^2 · dmol^{-1} (28). Molar ellipticities were calculated on a per residue basis using: a molecular weight of 162 per glycosyl residue but using only those residues in the galactomannan chain to facilitate comparison between

pPGM and PGM (the amount of carbohydrate present in the pPGM sample was multiplied by 365/385, whereas the value determined for PGM was used directly); and a molecular weight of 100 per amino acyl residue as there are about 30 residues in the 3,000 dalton polypeptide (8). Spectra were averaged point-by-point and then smoothed.

RESULTS

The CD of aqueous solutions of pPGM measured to 182.5 nm reveals two bands of negative ellipticity, one manifest as a shoulder near 220 nm and the other as a minimum at 200 nm (Fig. 1). The uncertainty in Fig. 1, 2000 deg · cm^2 · $dmol^{-1}$, is a result of high absorbance and the peptide constituting only 5% of the sample. No difference was seen in either peak position or intensity in 28.6% aqueous TFE (data not shown).

The CD of pPGM films is measured to 150 nm (Fig. 2, solid line). It exhibits a minimum at 217 nm of about half the intensity of that seen in solution. 200 nm is no longer an extremum. This ellipticity is nearly an order of magnitude less than that observed in solution. In the vacuum region a single asymmetric positive band is observed.

PGM showed no CD above 190 nm in solution and films as expected for a peptide-free preparation. The CD of PGM films is shown in Fig. 2 (dashed line). A single, nearly symmetrical, positive band is observed. The peak is shifted to lower energy compared to that seen in pPGM films.

Films of pPGM and PGM were clear and brittle (cellophane-like). All films used had no orientation as determined by a 90 degree rotation about the optical axis; however, variations in film thickness sometimes resulted in varying intensity of ellipticity at different film orientations. This variation in thickness is the cause of some of the uncertainty in the molar ellipticities reported in Fig. 2; the total uncertainty is approximately 20%.

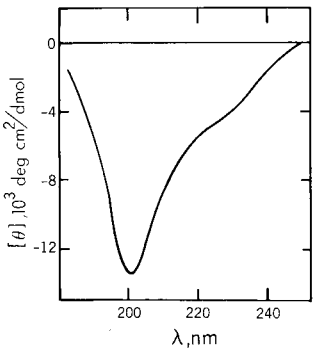

FIGURE 1

CD of pPGM in water. Molar ellipticity is per amino acyl residue (see Experimental Procedures).

DISCUSSION

Linear combinations of Greenfield & Fasman's (12) reference spectra do not yield any composites resembling the solution spectrum of pPGM (Fig. 1). The extended structure identified by Rinaudo & Domard (29) exhibits CD similar to that used to represent random coil by Greenfield & Fasman, with a small maximum at 220 nm and a larger minimum at 199 nm. They identify a different unordered form which exhibits a negative shoulder at around 220 nm and a minimum at 200 nm of lesser intensity than the 199 nm minimum of the extended structure. This unordered reference spectrum, referred to as "random" in their papers, is similar in intensity and shape to our spectrum

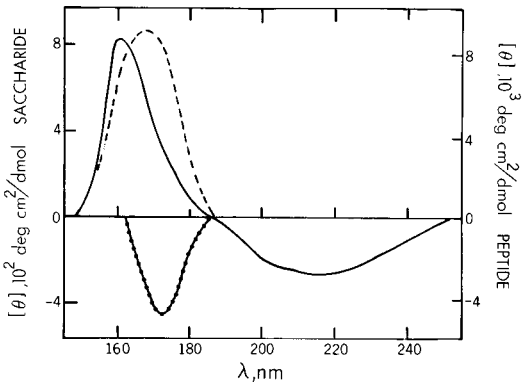

FIGURE 2

CD of pPGM film (solid line); CD of PGM film (dashed line); and difference CD (dotted line from 160 to 185 nm, solid line above 185 nm). Molar ellipticity per carbohydrate residue of the galactomannan is shown on the left ordinate and molar ellipticity per amino acyl residue is shown on the right ordinate.

of pPGM in solution above 195 nm (Fig. 1). A linear combination of 90% unordered and 10% β-sheet agrees better above 200 nm, but shows a more rapid rise to the baseline than is observed. Combination of this unordered reference spectrum with those of β-turns (30) results in the intensity of the 200 nm signal being significantly reduced from that observed. Fasman *et al.* (31) identify a CD spectrum which is characteristic of both an unordered, but tightly packed conformation in films, where there is little freedom for long range motion, and in denatured proteins, where repeating secondary structure has been disrupted but tight packing, maintained by disulfide bonds and/or non-covalent forces, limits long range freedom of motion. Their spectra are characterized by a negative shoulder from around 230–215 nm and a minimum near 200 nm. These features and their order of magnitude are in reasonable agreement with those of pPGM's solution spectrum to 195 nm (Fig. 1). There is a steeper rise toward the baseline in the reference spectra below 195 nm, as was also seen in comparison to the spectra of Rinaudo & Domard.

We conclude that the solution CD spectrum of pPGM (Fig. 1) reflects a peptide chain which is unordered but possesses some rigidity. The rigidity could be imposed by a folding of the peptide chain into a compact structure or perhaps by the glycosylation of approximately one-third of the residues. It has not yet been established whether glycosylation can result in sufficient chain stiffening to produce the observed CD. With regard to the proposed correlation between glycosylation and β-turns, we find no explicit contribution of β-turns in the solution CD of pPGM even though the polypeptide contains two prolyl residues. The two prolyl residues could increase the likelihood of a compact structure, or could contribute to the rigidity of an extended structure. Because there is no evidence for β-turns, it is more likely that the molecule is best described as a hydrated coil with some stiffness, rather than having any compact structure.

There is no alteration in the CD of pPGM in 28.6% TFE. Organic solvents, such as TFE, can act by decreasing the interactions of a molecule with water, thus increasing the probability of forming ordered structures which involve intramolecular hydrogen bonding. Twenty five percent ethanol has been observed to cause little change in the CD of native proteins, whereas 50–70% ethanol usually had a noticeable effect. However, 25% ethanol was sufficient to promote a change in the CD of denatured forms of ribonuclease which was interpreted as an increase in ordered structure (20). The absence of change in the CD of the peptide of pPGM may indicate inaccessibility of solvent to peptide resulting from the presence of saccharide side chains, preclusion of transition to a more ordered form by unfavourable kinetics, or insufficient intramolecular stabilizing forces for ordered forms of this relatively short peptide.

In films of pPGM (Fig. 2, solid line) there appear to be at least two peptide transitions present in the far u.v. as judged by the large width of the region of negative ellipticity. The ellipticity at 200 nm, however, is reduced nearly an order of magnitude from that seen in solution. The change in CD in going from solution to film could be due to a conformational change, a reweighting of statistical factors, or intermolecular packing interactions. Linear combinations of reference solution spectra do not yield the observed far u.v. film spectra of pPGM. Though film reference spectra have not been unified into a basis set, quantitative film spectra for β-sheets and random structures, as well as qualitative spectra of α-helices, are available (31, 32). The intensity of the CD signal in those films is greater than that seen in pPGM films. Our CD data of pPGM films cannot be decomposed, and it is doubtful that a richer basis set would allow such a decomposition, given the observed broad, weakly negative band.

At least one saccharide transition of the phosphogalactomannan is seen in the vacuum region with a maximum near 165 nm (Fig. 2, dashed line). Its low intensity compared to that of other polysaccharides could be caused by cancellation among the three classes of saccharide present, α–1→2 and α–1→6 linked mannosyl and β–1→5 linked galactofuranosyl residues, or by cancellation due to a lack of long range order, as many of the glycosidic linkages have freedom of rotation about two bonds. These two possibilities might be distinguished

through VUCD studies of additional pPGM derivatives.

Subtraction of PGM's film CD spectrum from that of pPGM leaves the contribution (Fig. 2, dotted line from 160–185 nm, solid line above 185 nm) of the peptide and its bound short mannose derivatives. A negative band is observed at 173 nm. Both peptide and saccharide electronic transitions occur near this wavelength, but it is of interest to note that a negative band near 170 nm is often observed in the CD of peptides (30, 32–34).

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