functions of plasminogen in haemostasis, such as its attachment to fibrin, binding of α_2-antiplasmin, and complexation with the preactivation peptide (Collen, 1980; Thorsen et al., 1981; Sjöholm et al., 1973). From this viewpoint, the aromatic signal identification achieved in the present study affords a sound basis for attempting a structural characterization of the kringles' lysine binding sites by NMR experimentation. Such investigations are in progress in our laboratories and their results shall be reported in due course.

Indole {N*H*}–C*H* NOE experiments on the BASA complex of kringle 4 performed after this paper was accepted for publication (A. Motta, R. A. Laursen, and M. Llinás, unpublished results) demonstrate unambiguously that the Trp-II doublet at 6.65 ppm corresponds to singlet 7 at 7.80 ppm (Figure 9), thus confirming the Trp-II (Trp-B) pairing scheme proposed by N. Pluck and R. J. P. Williams on the basis of paramagnetic lanthanide ion titration experiments. This identification leads to assigning the Trp^{72} singlet 1 to Trp-III (Trp-C).

Registry No. His, 71-00-1; Trp, 73-22-3; Tyr, 60-18-4; Phe, 63-91-2; plasminogen, 9001-91-6.

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Saccharomyces cerevisiae Structural Cell Wall Mannoprotein†

Jürgen Frevert‡ and Clinton E. Ballou*

Department of Biochemistry, University of California, Berkeley, California 94720

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ABSTRACT: A novel mannoprotein fraction with an average molecular weight of 180 000 has been isolated from *Saccharomyces cerevisiae mnn9* mutant cell wall that was solubilized by β-glucanase digestion. The same material could be extracted from purified wall fragments with 1% sodium dodecyl sulfate. The protein component, 12% by weight, is rich in proline, whereas the carbohydrate, mainly mannose, is about evenly distributed between asparagine and hydroxyamino acids. Endoglucosaminidase H digestion of the isolated mannoprotein reduced its average molecular weight to 150 000, but the mannoprotein, while still embedded in the cell wall, was inaccessible to the enzyme. Biosynthesis and translocation of the mannoprotein were investigated by following incorporation of [^{3}H]proline into this fraction. In the presence of tunicamycin, both *mnn9* and wild-type X2180 cells made a mannoprotein fraction with an average molecular weight of 140 000, whereas in the absence of the glycosylation inhibitor, the *mnn9* mutant made material with a molecular weight of 180 000 and the mannoprotein made by wild-type cells was too large to penetrate the polyacrylamide gel. Although the cell wall mannoprotein was resistant to heat and proteolytic enzymes, attempts to isolate the carbohydrate-free component failed to yield any characteristic peptide material.

The yeast cell envelope has been postulated to contain at least three classes of mannoprotein: the *inducible hydrolytic enzymes*, such as external invertase and acid phosphatase, the *sexual agglutinins*, expressed on cells that are homozygous at the mating-type locus, and, lacking a better term, *structural mannoproteins* (Ballou, 1976). The external invertase of *Saccharomyces cerevisiae* (Gascón et al., 1968; Tarentino et al., 1974; Lehle et al., 1979) and the sexual agglutinins of *Hansenula wingei* (Crandall & Brock, 1968; Yen & Ballou, 1974; Burke et al., 1980) have been purified and analyzed in some detail, but little is known about the presumed structural mannoprotein other than what has been learned fortuitously from analyses performed on the total cell mannoprotein (Phaff, 1971; Ballou, 1976; Cohen et al., 1982).

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‡Present address: Battelle-Institute, Romerhof 35, 6000 Frankfurt, West Germany.

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A new mutant of *S. cerevisiae*, designated *mnn9*,[1] was recently isolated that synthesizes mannoproteins with truncated carbohydrate chains (Ballou et al., 1980). The invertase made in this mutant has a subunit molecular weight of about 92 000 (Frevert & Ballou, 1982), whereas the wild-type invertase is about 120 000 (Gascón et al., 1968). We now find that the cell wall of this mutant contains a mannoprotein fraction with an average molecular weight of 180 000. The carbohydrate component is about half asparagine linked and half linked to the hydroxyamino acids, whereas the protein is rich in proline, which along with serine, threonine, aspartic and glutamic acids, glycine, and alanine makes up 87% of the amino acids. The unusual constitutive mannoprotein fraction we now call the "cell wall mannoprotein" (CWMP),[1] and our studies suggest that it is also present in the wild-type cell wall but in a form too large to penetrate a 7% polyacrylamide gel owing to the longer carbohydrate chains linked to asparagine. The uniform size and general composition of the mannoprotein are consistent with a structural role in the cell wall.

Experimental Procedures

Materials. *S. cerevisiae* X2180 was from the Yeast Genetic Stock Center, University of California, Barkeley, whereas the *mnn2 mnn9* mutant was isolated to this laboratory (Ballou et al., 1980). Endo-*N*-acetyl-β-D-glucosaminidase H was purified according to Tarentino et al. (1978) and α-mannanase according to Jones & Ballou (1969), and zymolyase 60 000 was obtained from Seikaguku (Tokyo, Japan). HTP-hydroxylapatite, Bio-Gel P-30 (−400 mesh), and Bio-Gel P-2 (50–100 mesh) were from Bio-Rad; Ultragels ACA 34 and ACA 54 were from LKB, whereas DEAE-Sephadex A-50, Cetavlon (hexadecyltrimethylammonium bromide), protease K, trypsin, chymotrypsin, subtilisin, and jack bean α-mannosidase were obtained from Sigma. L-[2,3,4,5-^{3}H]Proline (100 Ci/mmol) was from Amersham.

Methods. Crude mannoprotein was prepared from *mnn2 mnn9* cells by extraction with hot citrate buffer followed by precipitation with methanol and then Cetavlon–borate (Nakajima & Ballou, 1974). The product was chromatographed on DEAE-Sephadex A-50 by elution with NaCl, and the material from this step was purified to homogeneity on a hydroxylapatite column. Mannoprotein was isolated from cell wall digests prepared as described by Schwenke & Nagy (1978). The protoplasts were removed and mannoprotein was isolated from the supernatant solution by chromatography on DEAE-Sephadex A-50 and on HTP-hydroxylapatite. Cell wall fragments were purified according to Fleet & Phaff (1973), and mannoprotein was extracted by boiling the fragments with 1% sodium dodecyl sulfate.

To release asparagine-linked oligosaccharides, mannoprotein was incubated with endoglucosaminidase H and the solution was chromatographed on an Ultragel ACA 54 column in 0.1 M ammonium acetate. The oligosaccharide peak was fractionated on a Bio-Gel P-4 column, whereas the recovered protein peak was analyzed by gel electrophoresis and for amino acid and hexosamine content.

Hexose was determined by the phenol–sulfuric acid procedure (Dubois et al., 1966), glucosamine according to Ghosh et al. (1960) after hydrolyzing the mannoprotein for 8 h in 4 N HCl at 110 °C under vacuum, and protein by the method of Lowry et al. (1951). Gel electrophoresis in sodium dodecyl sulfate was done according to Laemmli (1970) on 7% gels.

[1] Abbreviations: PAS, periodic acid–Schiff reagent; SDS, sodium dodecyl sulfate; CWMP, cell wall mannoprotein; *mnn*, designator for a mannoprotein mutation; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

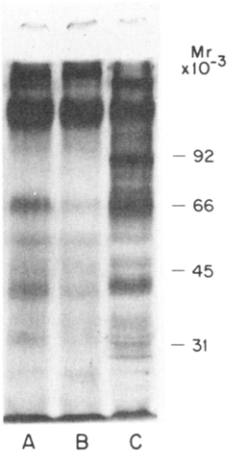

FIGURE 1: Preliminary detection of a cell wall mannoprotein by SDS gel electrophoresis of the *mnn2 mnn9* cell wall solubilized by zymolyase digestion. (A) Cells were grown on glucose and the extract was boiled 10 min with SDS; (B) as in (A), except the extract was not boiled; (C) cells were derepressed with sucrose and the extract was boiled 10 min with SDS. Molecular size markers were carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (66 000), and phosphorylase B (92 000). The CWMP is the major band near the top of the gel (M_r 180 000), whereas invertase migrates at about M_r 92 000. Two sharp bands in the M_r 200 000–220 000 region are also apparent. The gel was stained with the PAS reagent.

The CWMP was not revealed by protein strains, but it could be stained by the periodic acid–Schiff reagent (Faribanks et al., 1971).

The protein component of mannoproteins was radiolabeled by incubating whole cells with [^{3}H]proline, and the material released from the cell wall by digestion of the cells with zymolyase was analyzed by gel electrophoresis in sodium dodecyl sulfate. In some experiments, tunicamycin (5 μg/mL) was added to inhibit glycosylation. Attempts were also made to obtain the carbohydrate-free mannoprotein by chemical deglycosylation using HF (Mort & Lamport, 1977) or trifluoromethanesulfonic acid (Edge et al., 1981).

Results

Preliminary Identification of a Cell Wall Mannoprotein. Cells of the *mnn2 mnn9* mutant were digested with zymolyase 60 000 in the presence of an osmotic stabilizer, and the intact protoplasts were removed by centrifugation (Schwenke & Nagy, 1978). The supernatant solution was then dialyzed and subjected to gel electrophoresis in the presence of sodium dodecyl sulfate. The results in Figure 1 are for cells repressed on glucose (lanes A and B) and for cells derepressed on sucrose (lane C). In all examples, the major band stained with the periodate–Schiff (PAS) reagent for carbohydrate migrates near the top of the gel (M_r about 180 000), and the derepressed cells show a band for invertase (M_r 92 000) of a size characteristic of the *mnn9* mutant (Frevert & Ballou, 1982). All extracts show some faster migrating bands that may have resulted from protoplast lysis and could be intracellular in origin. The gel also shows two sharp bands (M_r 200 000 and 220 000) that migrate slower than the major mannoprotein, and the intensities of these two bands differ in the repressed and derepressed cell extracts.

Attempts to demonstrate a major cell wall mannoprotein in *S. cerevisiae* X2180 were unsuccessful because the mannoprotein material in extracts such as those described above failed to enter the gel during electrophoresis. This is probably due solely to the higher carbohydrate content of the wild-type mannoproteins, although there could be some other form of cross-linking, dependent on the presence of the mannoprotein

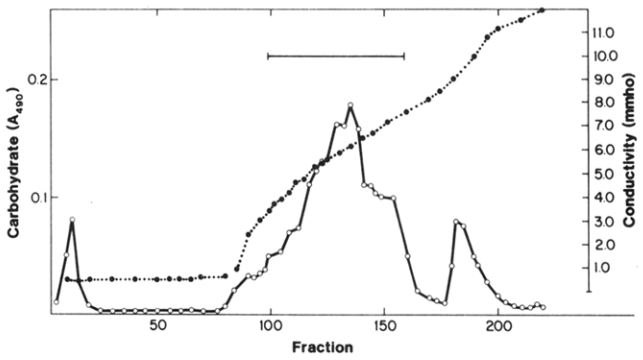

FIGURE 2: Purification of the CWMP by DEAE-Sephadex A-50 chromatography. The partially purified mannoprotein was applied in 10 mM Tris-HCl buffer, and the mannoprotein was eluted with a 0–300 mM NaCl gradient. Carbohydrate (A_{490}) and conductivity are shown. The CWMP fractions were combined as indicated by the bar.

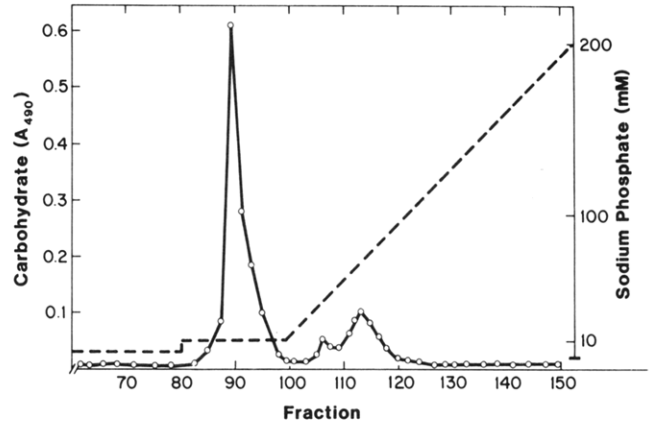

FIGURE 3: Purification of the CWMP by HTP-hydroxylapatite chromatography. The material recovered from the DEAE-Sephadex A-50 column was applied to a hydroxylapatite column in 2 mM sodium phosphate and eluted with 10 mM sodium phosphate. Carbohydrate (A_{490}) and the sodium phosphate concentrations are shown. The material in fractions 87–95 was combined.

outer chain, that would make the molecules too large to penetrate the gel.

Purification of the Cell Wall Mannoprotein from mnn2 mnn9 Cells. From 180 g of a 48-h culture of cells, extracted with citrate buffer and precipitated as the Cetavlon–borate complex, was obtained 675 mg of carbohydrate in the form of mannoprotein (Nakajima & Ballou, 1974). This material was dissolved in dilute acid, the mannoprotein was precipitated with methanol, the precipitate was dissolved in 85 mL of 10 mM Tris-HCl buffer, pH 7.5, and the solution was applied to a DEAE-Sephadex A-50 column (4 × 15 cm). The column was eluted with a linear gradient of 0–300 mM NaCl in 10 mM Tris-HCl, pH 7.5, and the mannoprotein appeared in a broad peak at a conductivity between 3.5 and 7.0 mmho (Figure 2). The yield of carbohydrate was 239 mg. Without dialysis, the combined fractions from the DEAE-Sephadex column were applied to a column (2 × 15 cm) prepared from 15 g of HTP-hydroxylapatite equilibrated with 2 mM sodium phosphate, pH 7.5. After elution of unbound material, the pure mannoprotein was eluted with 10 mM sodium phosphate, pH 7.5 (Figure 3). The yield was 186 mg of carbohydrate. Gel electrophoresis of the mannoprotein is shown in Figure 4, lane A, at the Cetavlon–borate stage and Figure 4, lane D, after hydroxylapatite chromatography. Although the band in lane D is broadened, the preparation lacks contaminants of a smaller or larger size, which suggests that it represents a specific mannoprotein type. The cell wall mannoprotein

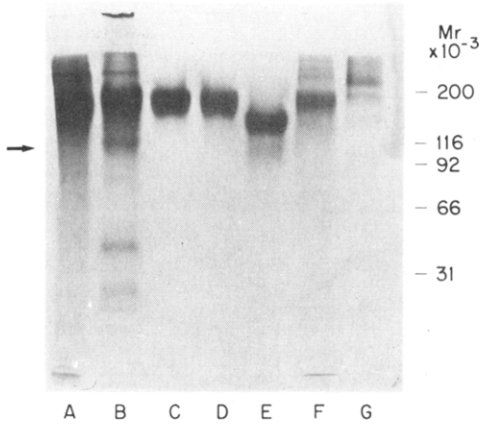

FIGURE 4: Gel electrophoresis in SDS of isolated *mnn2 mnn9* mannoprotein fractions. (A) Material precipitated as the Cetavlon–borate complex after citrate buffer extraction; (B) supernatant fraction from derepressed cells treated with zymolyase for preparation of protoplasts; (C) CWMP purified from (B) through the hydroxylapatite step; (D) CWMP purified from whole cells through the hydroxylapatite step; (E) CWMP from (D) after treatment with endoglucosaminidase H; (F) purified cell wall fragments extracted with 1% SDS; (G) purified cell wall fragments extracted with 10 mM dithioerythritol. The molecular size markers were as in Figure 1, plus β-galactosidase (116000) and myosin (200000). The gel was stained with the PAS reagent.

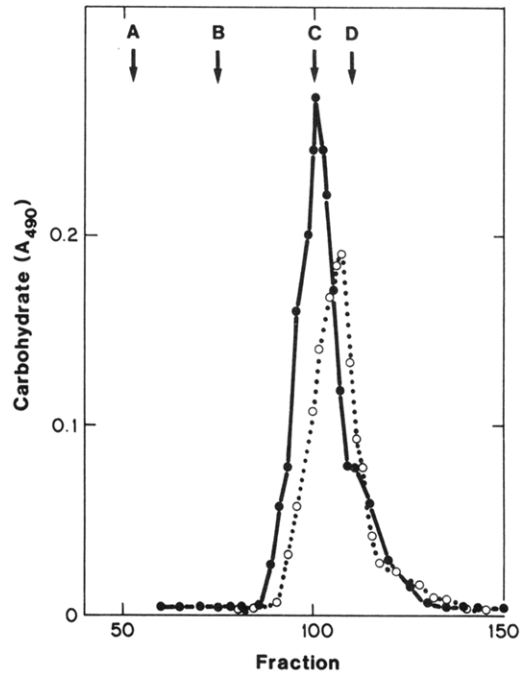

FIGURE 5: Sizing of mannoproteins by gel filtration on Ultragel ACA 34. The purified CWMP was chromatographed in 10 mM Tris-HCl, pH 7.5, containing 100 mM NaCl before (solid line) and after (dashed line) digestion with endoglucosaminidase H. The void volume of the column (A) and the positions of elution of invertase dimer [(B) 240000], fumarase tetramer [(C) 192000], and aldolase [(D) 160000] are indicated.

could be stained on gels only with the PAS stain, and no contaminant that stained with Coomassie blue or a silver stain (Morrissey, 1981) was present. The broad diffuse band seen on electrophoresis was independent of the amount applied to the gel.

Gel electrophoresis of the cell wall mannoprotein indicated that it had an average M_r of 180000. When chromatographed on Ultragel ACA 34, the mannoprotein ran as a monomer near the position of fumarase, which has a M_r of 192000 (Figure 5). The purified material contained 12% protein (Lowry et

Table I: Composition of Cell Wall Mannoprotein

preparation	mannose content (mg/mg of protein)[a]	hexosamine content (μmol/mg of protein)[a]
cell wall mannoprotein	7.62	1.29
Endo H digested CWMP	4.68	0.66

[a] Protein was by the Lowry procedure (Lowry et al., 1951).

al., 1951) and 88% carbohydrate (Dubois et al., 1966) (Table I).

To prepare mannoprotein from cell wall digests, 53 g of cells was incubated with zymolyase 60 000 (Schwenke & Nagy, 1978), after which the intact protoplasts were removed by centrifugation at 4000 rpm for 20 min and were washed with the incubation buffer by a second centrifugation. To remove most proteins, solid ammonium sulfate was added to the combined dialyzed supernatant fractions to a concentration of 75% of saturation, and the precipitate was removed by centrifugation and discarded. The supernatant solution was dialyzed for 24 h against distilled water, after which it was adjusted to pH 7.5 and subjected to chromatography on DEAE-Sephadex A-50 and on HTP-hydroxylapatite as described above to yield 55 mg of mannoprotein as carbohydrate. Gel electrophoresis of this mannoprotein preparation is shown in Figure 4, lane B, for the initial supernatant after removal of the protoplasts and in Figure 4, lane C, after the hydroxylapatite column step. The apparent identity between the preparations from the whole cells and from the cell wall digest clearly localizes the mannoprotein in the extracellular compartment. Lane B of Figure 4 also shows the presence of external invertase (the cells had been derepressed by growth on sucrose) and of a sharp band that migrates slower than the major mannoprotein band. Both of these components are removed during the purification steps.

Finally, cell wall fragments prepared with a Braun homogenizer were purified (Fleet & Phaff, 1973), and 10 mg was extracted with 1 mL of boiling 1% sodium dodecyl sulfate in 50 mM Tris-HCl buffer, pH 6.8, to yield 375 μg of solubilized carbohydrate. Gel electrophoresis of this extract is shown in Figure 4, lane F, and the major band is seen to correspond to the cell wall mannoprotein, which confirms that the material is localized in the wall itself. Extraction of these same cell wall fragments with 10 mM dithioerythritol in 50 mM Tris-HCl, pH 8.8, a treatment used to "activate" cells before digestion with β-glucanase to solubilize the wall (Schwenke & Nagy, 1978), contained very little if any of the cell wall mannoprotein (lane G), but it did contain some more slowly migrating material that may correspond to the slower band in lanes A, B, and F. This same band is also observed in dithioerythritol extracts of whole cells (see below), which fact could reflect a surface location for this material.

Endoglucosaminidase Digestion of the Cell Wall Mannoprotein. Mannoprotein prepared from whole cells by citrate extraction (190 mg of carbohydrate) was incubated with 30 milliunits of endoglucosaminidase H for 36 h at 37 °C in 1 mL of 50 mM citrate buffer, pH 5.5. The solution was chromatographed on an Ultragel ACA 54 column (2 × 50 cm) in 0.1 M ammonium acetate. The recovered protein peak was dialyzed, lyophilized, and digested a second time with endoglucosaminidase H in the same manner, after which a third digestion even in the presence of sodium dodecyl sulfate failed to release any more carbohydrate. The product was fractionated on the Ultragel ACA 54 column, and the combined oligosaccharide fraction was resolved according to size on a Bio-Gel P-4 column (2 × 200 cm) by elution with water. The pattern is shown in Figure 6. The structures of these oligosaccharides are reported (Tsai et al., 1984), and they correspond to $Man_xGlcNAc$ in which $x = 11-14$.

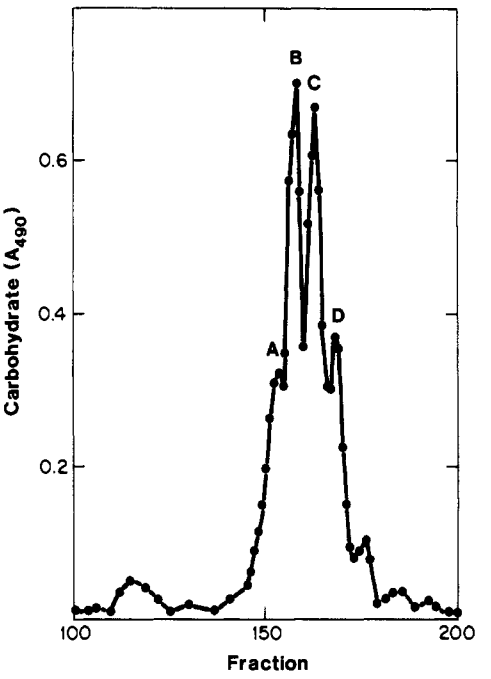

FIGURE 6: Fractionation of oligosaccharides released from CWMP by endoglucosaminidase H. The oligosaccharides were separated according to size on a Bio-Gel P-4 column by elution with water. (A) $Man_{14}GlcNAc$; (B) $Man_{13}GlcNAc$; (C) $Man_{12}GlcNAc$; (D) $Man_{11}GlcNAc$ (Tsai et al., 1984).

Table II: Amino Acid Composition of Cell Wall Mannoprotein

amino acid[a]	residues/100 amino acids	amino acid[a]	residues/100 amino acids
threonine	30.3	alanine	9.1
serine	14.9	valine	3.2
aspartate/asparagine	8.2	isoleucine	2.9
glutamate/glutamine	8.6	leucine	2.1
proline	11.0	tyrosine	2.0
glycine	5.2	phenylalanine	1.4

[a] Less than 0.5 residue/100 amino acids of tryptophan, histidine, lysine, and arginine was observed, and no cysteine or methionine was detected.

The yield of released oligosaccharides was 48 mg and that of the residual mannoprotein was 108 mg as carbohydrate, which represents an 83% recovery of the carbohydrate. The composition of the recovered protein peak is given in Tables I and II, and its gel electrophoresis is shown in Figure 4, lane E. Endoglucosaminidase digestion reduced the hexosamine content by half, as expected if all of the asparagine-linked units had been released. The remaining carbohydrate must be linked to serine and threonine because it was released by alkaline conditions that promote β-elimination, and the product consisted of a mixture of mannose, mannobiose, mannotriose, and mannotetraose in the molar ratio 1:5:4:4 (Nakajima & Ballou, 1974). A reduction in size of the mannoprotein following endoglucosaminidase H digestion was also demonstrated by gel filtration on Ultragel ACA 34 (Figure 5). An unusual assortment of seven amino acids makes up the bulk of the protein component (Table II), which is probably blocked at its N terminus because an attempt to identify the amino terminal gave a negative result (data not shown).

Chemical Deglycosylation of Mannoprotein. Extensive digestion of the residual mannoprotein from above with jack bean or bacterial α-mannosidases removed only a small part of the O-linked mannose, so attempts were made to use chemical procedures for this purpose. Desalted, lyophilized,

Table III: Incorporation of [^{3}H]Proline by Whole Cells

extract	radioactivity (cpm × 10^{-6}) in wild-type cells	wild-type + tunicamycin	*mnn2 mnn9* cells	*mnn2 mnn9* cells + tunicamycin
dithioerythritol extract	2.2	2.1	7.2	7.2
protoplast supernatant extract	7.9	6.2	10.9	9.1
shocked protoplast extract	32.4	40.4	25.2	35.6

endoglucosaminidase H digested mannoprotein (55 mg of carbohydrate) was treated with 5 mL of HF (dried over CoF_3) in the presence of 2.5 mL of anisole for 2 h at 0 °C (Mort & Lamport, 1977). After evaporation of the HF and anisole, the brown gummy residue was dissolved in 50% acetic acid and passed through a Bio-Gel P-2 column. The material that appeared in the void volume was free of carbohydrate and was analyzed by electrophoresis or gel filtration on Bio-Gel P-30, but no characteristic protein component was observed.

Deglycosylation with trifluoromethanesulfonic acid was done according to Edge et al. (1981). To 10 mg of mannoprotein were added 0.8 mg of anisole and 3.5 mL of trifluoromethanesulfonic acid, both freshly distilled. The air was displaced with He, and the tube was sealed with a rubber stopper. The slightly yellow solution was sonicated for 30 min at 0 °C and kept at the same temperature for 1–8 h. The solution was then cooled to −20 °C and 7 mL of 50% pyridine in water was added dropwise until the pH was about 7. The solution was extracted 3 times with 1-mL portions of ether. The product was fractionated on a Bio-Gel P-2 column, and the large excluded material representing the protein part was analyzed by gel electrophoresis. Again, no distinct band representing a protein component was seen.

Labeling of Cell Wall Mannoprotein in Whole Cells. A suspension of *mnn2 mnn9* or wild-type cells grown for 12 h in minimal medium plus 0.5 M KCl (10 A_{600} units) was washed with 40 mM sodium phosphate, pH 6.0, containing 0.5 M KCl and then incubated with 0.75 mL of 40 mM sodium phosphate, pH 6.0, containing 0.5 M KCl, 1% glucose, and 0.25 mCi of [^{3}H]proline for 2 h at 30 °C. To study the effect of N-glycosylation inhibition, cells were preincubated with 5 μg/mL tunicamycin in the same buffer for 20 min at 30 °C prior to the addition of the labeled proline.

After labeling, the cells were washed twice with 2.5 mL of 0.5 M KCl and then treated for 20 min with 120 mM dithioerythritol in 1 mM EDTA and 100 mM Tris-HCl buffer, pH 8.8 (dithioerythritol extract). The cells were washed twice with 2-mL portions of 50 mM 2-(*N*-morpholino)ethanesulfonic acid–KOH, pH 6.0, containing 0.6 M sorbitol, and then were digested with 0.5 mg of zymolyase in 1 mL of 50 mM Tris-HCl, pH 7.2, containing 1.0 M KCl and 1 mM dithioerythritol for 60 min at 30 °C, to solubilize the walls. The protoplasts were removed by centrifugation of the solution at 4000 rpm for 10 min and were washed with 1 mL of the same buffer. The combined supernatant liquid and washing are the "protoplast supernatant". The protoplasts were then lysed in 1 mL of 50 mM Tris-HCl, pH 7.5, and the insoluble material was removed by centrifugation to yield the "shocked protoplast supernatant". The fractions were exhaustively dialyzed, and the radioactivity found in each is listed in Table III.

Portions of each protoplast supernatant extract, containing about 200 000 cpm of ^{3}H radioactivity, were analyzed by gel electrophoresis. The bulk of the radioactivity in these fractions

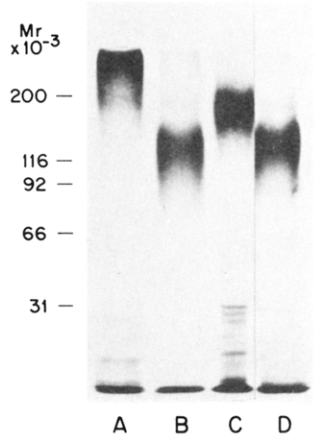

FIGURE 7: Effect of tunicamycin on glycosylation of CWMP. Supernatant extracts from cells digested with zymolyase were analyzed by SDS gel electrophoresis after removal of the protoplasts by centrifugation. (A) Wild-type CWMP and (C) *mnn2 mnn9* CWMP from cells incubated with [^{3}H]proline in the absence of tunicamycin; (B) wild-type CWMP and (D) *mnn2 mnn9* CWMP from cells incubated with [^{3}H]proline in the presence of tunicamycin. Molecular size markers are as in Figures 1 and 4. The bands were detected by autoradiography.

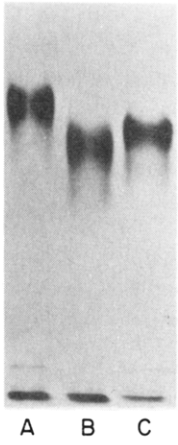

FIGURE 8: Comparison of the effect of tunicamycin and endoglucosaminidase H on *mnn2 mnn9* CWMP size. SDS gel electrophoresis of CWMP from cells incubated with [^{3}H]proline. (A) In the absence of tunicamycin; (B) in the presence of tunicamycin; (C) CWMP from (A) after digestion with endoglucosaminidase H. The bands were detected by autoradiography.

was found in the cell wall mannoprotein (Figure 7) and, as expected, tunicamycin reduced the apparent molecular size of both the wild-type and *mnn2 mnn9* mannoproteins to materials with an average M_r of 140 000 (parts B and D of Figure 7), somewhat smaller than that found on digestion of the *mnn2 mnn9* mannoprotein with endoglucosaminidase H (Figure 8). The failure of the mannoprotein, made in wild-type cells in absence of tunicamycin, to enter the gel demonstrates that its size must be in excess of M_r 220 000.

The accessibility of the labeled mannoprotein in the intact cell wall to digestion by endoglucosaminidase H was examined. As shown in Figure 9, the apparent size of the mannoprotein, judged by gel electrophoresis, was not altered in either the dithioerythritol or protoplast supernatant extracts. As in Figure 4, the labeled material extracted by dithioerythritol (D–F) has a larger apparent size than the CWMP (A–C), although it is heavily labeled by [^{3}H]proline.

Discussion

Several previous studies have dealt with attempts to characterize the cell wall mannoprotein of *S. cerevisiae*. Sentan-

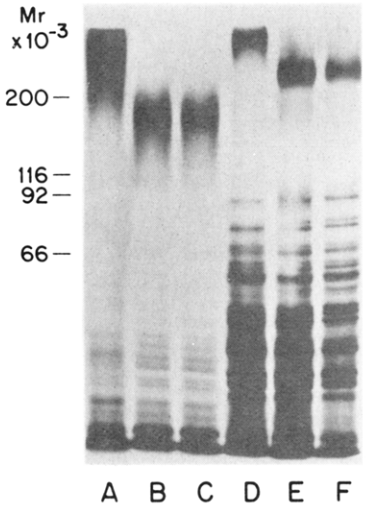

FIGURE 9: Endoglucosaminidase H digestion of intact cells. Cells were incubated with [^{3}H]proline to label the CWMP and then were digested with endoglucosaminidase H. Supernatant extracts of cells digested with zymolyase after the following treatments: (A) wild-type cells and (B) *mnn2 mnn9* cells digested with endo H; (C) *mnn2 mnn9* cells without endo H digestion. Dithioerythritol extracts of whole cells after the following treatments: (D) wild-type cells and (E) *mnn2 mnn9* cells digested with endo H; (F) *mnn2 mnn9* cells without endo H digestion. The bands were detected by autoradiography.

dreau & Northcote (1968) described a wall mannoprotein from bakers' yeast, extracted with ethylenediamine, that had a M_r of 76 000 and contained 4% protein, whereas material isolated by Eddy & Longton (1969), following glucanase digestion, had a M_r of 189 000–330 000. In studies with the *mnn2* mutant of *S. cerevisiae*, which makes asparagine-linked polymannose chains that are unbranched in the order chain (Ballou, 1976), Nakajima & Ballou (1974) prepared mannoprotein by the borate–Cetavlon procedure that had a M_r of 133 000 and that was reduced to about 40 000 after treatment with alkali. Shibata et al. (1983) have recently reported a similar molecular weight for the same mannoprotein. Thus, although there has been some suggestion that the yeast cell wall might contain a characteristic mannoprotein, there was no clear evidence that the material might be relatively homogeneous with regard to size. The novel feature of our study is the demonstration that this is so, and the observation was dependent on the availability of the new *mnn9* mutant (Ballou et al., 1980). Because this mutant makes and secretes mannoprotein with truncated outer chains, the reduced size allows it to penetrate the gel during electrophoresis. In contrast, the cell wall mannoprotein secreted by *S. cerevisiae* X2180 does not enter the gel and cannot be analyzed in this way, except for cells grown in the presence of glycosylation inhibitors.

We have called this mannoprotein fraction the "cell wall mannoprotein". It contains about 12% protein and 88% carbohydrate. The CWMP is not revealed on gels by any protein stain we tried, but it is stained by the PAS reagent (Fairbanks et al., 1971) and can be estimated in solution by the Lowry method (Lowry et al., 1951). The carbohydrate component of *S. cerevisiae* mannoproteins is all D-mannose except for the small amount of *N*-acetyl-D-glucosamine that is involved in the saccharide–protein linkage. Approximately half of the mannose is attached to asparagine in the mannoprotein and the other half to serine and threonine.

The protein component of the mannoprotein is unusual in its high content of proline and in the low level or absence of the basic and sulfur-containing amino acids. The high content of serine and threonine is typical of extracellular yeast mannoproteins (Ballou, 1976) and is consistent with the large amount of mannose that is attached to these amino acids. A similar amino acid composition was reported by Shibata et al. (1983) for the mannoprotein from the *mnn2* mutant. The precise nature of the protein component or its degree of heterogeneity could not be determined because we were not successful in removing enough of the carbohydrate to permit further analysis. Even after endoglucosaminidase H and exomannanase digestion, the product retained a large amount of carbohydrate and resisted attack by all proteases investigated, whereas attempted chemical deglycosylation led to extensive degradation of the protein. The mannoprotein could be extracted from purified cell wall fragments by hot 1% sodium dodecyl sulfate but not by 10 mM dithioerythritol. Thus, it does not appear to be cross-linked in the wall by disulfide linkages.

Tunicamycin inhibited N-glycosylation of the cell wall mannoprotein, but the product made under these conditions still contained carbohydrate, probably linked to serine and threonine since much of it was released by β-elimination. Because the altered glycoprotein was found in the cell wall, it is clear that the inhibitor did not prevent translocation. The mannoprotein made in wild-type cells in the presence of tunicamycin was reduced to the same size as that made in *mnn2 mnn9* cells incubated with the inhibitor, which indicates that all N-glycosylation was prevented under these conditions and that the secreted material contained only O-linked mannose.

Advantage was taken of the high content of proline in the mannoprotein to investigate its biosynthesis. The cell wall material was readily labeled when cells were incubated with [^{3}H]proline, and the effect of tunicamycin on the size of the mannoprotein made in its presence was confirmed. In this instance, however, the mannoprotein made in the *mnn2 mnn9* mutant in the presence of tunicamycin was somewhat smaller than that obtained by treating the *mnn2 mnn9* mannoprotein with endoglucosaminidase H, which suggests that the enzyme does not remove all of the N-linked carbohydrate. Alternatively, the slightly larger size of the latter product could be due to the asparagine-linked *N*-acetylglucosamine that remains with the protein after enzyme digestion (Huffaker & Robbins, 1983). The ready accessibility of the asparagine-linked chains to endoglucosaminidase H in the isolated mannoprotein does not extend to the mannoprotein in the cell wall, because the ^{3}H-labeled mannoprotein of intact cells was not attacked by this enzyme.

In cells that were derepressed for invertase, a band for this glycoprotein (M_r 92 000) was seen on gel electrophoresis. A sharp band that stained for carbohydrate was also seen at a position of M_r 200 000 in these extracts, whereas in the repressed cells there was a band at about M_r 220 000 and only a minor band at M_r 200 000. Both these high molecular weight components and the invertase are removed during purification of the cell wall mannoprotein. It is possible that the two high molecular weight bands are present in the wild-type extract but are hidden beneath the diffuse CWMP band at the origin. Therefore, we do not know whether the *mnn9* mutation alters the properties of these two bands, although the fact that they react with the PAS stain indicates that they are glycoproteins.

Pastor et al. (1982) have investigated the turnover kinetics of periplasmic and wall mannoproteins in *S. cerevisiae* by pulse–chase experiments. Because gel electrophoresis was not applied to the various fractions they analyzed, the changes observed must reflect an average metabolic flux of all of the components in each fraction. They noted, however, that the wall-associated mannoproteins had a slow rate of turnover relative to the periplasmic mannoproteins, and it seems

probable that the stable component consists mainly of the CWMP we have identified.

A recent study (Novick & Schekman, 1983) has dealt with the identification of *S. cerevisiae* cell wall components that become labeled, either chemically by iodination of intact cells with iodogen–$Na^{125}I$ or metabolically by incubation of whole cells with $^{35}SO_4^{2-}$. Analysis of the wall components by SDS gel electrophoresis, after solubilization by treatment with β-glucanase, revealed several labeled bands. Because iodination depends on the presence of tyrosine residues, which are found in CWMP, and sulfur labeling on the presence of cysteine or methionine residues, both of which are very low or absent in the cell wall mannoprotein studied here, there is no reason to expect a quantitative correspondence between any of these labeled bands and the CWMP detected by the PAS stain. It seems likely, however, that labeled material in the extract from strain X2180 described by Novick & Schekman (1983) as failing to migrate beyond the 5% gel may correspond to the CWMP we have characterized.

Registry No. Mannose, 3458-28-4.

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