Mannan-protein Location and Biosynthesis in Plasma Membranes from the Yeast Form of *Candida albicans*

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Specific labelling of the plasma membrane of intact protoplasts from *Candida albicans*, using lactoperoxidase-catalysed iodination, has permitted the development of a procedure for isolating relatively pure preparations of this component. The specific activity of the labelled membrane was very low, indicating that only a few proteins are exposed on the outer membrane surface. The specific activity of labelling increased almost 100-fold when both membrane surfaces were exposed to iodination. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, in combination with autoradiography, indicated that only two glycosylated proteins are exposed on the outer membrane surface, whilst all membrane proteins can be labelled in isolated plasma membrane preparations. Extraction of mannan-protein from purified cell walls of *C. albicans* gave material which, on sodium dodecyl sulphate-polyacrylamide gel electrophoresis, resembled the exposed plasma membrane glycoproteins.

Purified plasma membranes incorporated mannose from GDPmannose on to an endogenous protein acceptor. The addition of exogenous cell wall mannan-protein increased the degree of incorporation. Approximately 12% of the incorporated mannose was sensitive to β -elimination, but a lipid intermediate was not involved in the reaction. An Arrhenius plot of enzyme activity at different temperatures showed a discontinuity at 17 °C. The mannan synthetase activity was also significantly less at 40 °C than at 37 °C, temperatures at which the yeast-mycelial transition has been found to occur in this organism. The possible biosynthetic relationship between the exposed glycoproteins of the plasma membrane and those of the cell wall is discussed.

INTRODUCTION

The use of non-penetrant covalent labels for 'tagging' plasma membranes has proved a valuable aid in the isolation of this component from a number of eukaryotic organisms (Wallach & Lim, 1973). The iodination of immunoglobulins by purified lactoperoxidase was first described by Marchalonis (1969) and this has provided a widely used technique for the specific radioactive labelling of plasma membranes from a number of organisms (Phillips & Morrison, 1970; Poduslo, Greenberg & Glick, 1972), including the yeasts *Saccharomyces cerevisiae* (Schibeci, Rattray & Kidby, 1973*a*; Duran, Bowers & Cabib, 1975) and *Candida albicans* (Marriott, 1975*a*). Lactoperoxidase-catalysed iodination of intact cells lacking a cell wall can also be used to demonstrate the asymmetric distribution of membrane proteins across the plane of the lipid bilayer (Poduslo *et al.*, 1972; Phillips & Morrison, 1971; Salton, Schor & Ng, 1972). However, the use of this technique and the interpretation of the results obtained are open to criticism unless stringent precautions are taken (Morrison, 1974).

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Following the reports of Schibeci *et al.* (1973*a, b*), it was found that relatively pure preparations of plasma membrane could be isolated from disrupted protoplasts of *C. albicans* using differential and discontinuous sucrose density gradient flotation centrifugation (Marriott, 1975*a*). The position of the plasma membrane on the gradients was determined by radioassay. The possibility therefore existed for identifying the plasma membrane proteins and studying their disposition in this organism. Chemical analysis indicated the presence of polysaccharides, perhaps as glycoproteins, which are enriched in mannose, compared with the cell wall (Marriott, 1975*a*).

The structure of baker's yeast cell wall mannan-protein has been studied extensively (Ballou, 1974; Nakajima & Ballou, 1974) and a complex enzymic mechanism for its biosynthesis has been proposed (Nakajima & Ballou, 1975). At least some of the enzymes involved, as well as the endogenous protein acceptor, appear to be membrane-bound (Lehle & Tanner, 1974; Sharma *et al.*, 1974). The type of membrane involved is unknown, although mannan synthetase activity has been found in purified preparations of plasma membrane from *C. albicans* (Marriott, 1975*b*). This paper presents evidence for the nature and disposition of the proteins in the plasma membrane of *C. albicans*. In addition the possible relationship between the plasma membrane and cell wall mannan-proteins has been studied together with the properties of the membrane-bound mannan synthetase.

METHODS

Organism and growth. *Candida albicans* 6406 was maintained and grown as described previously (Marriott, 1975*a*). Cells were harvested in the mid-exponential phase of growth and protoplasts were prepared using a lytic enzyme preparation from *Streptomyces violaceus* with 1.2 M-sorbitol (final concentration) as osmotic stabilizer.

Iodination. A modification of the procedure described by Marriott (1975*a*) was used in an attempt to increase the specific activity of the product. Protoplasts were resuspended in 5 ml 50 mM-citrate/phosphate buffer, pH 7.4, containing 1.2 M-sorbitol, at a density equivalent to 100 mg dry wt cells ml⁻¹. Lactoperoxidase (Sigma) and Na¹²⁵I (The Radiochemical Centre, Amersham) made up in 10 μ M-Na₂SO₃ were added to final concentrations of 0.2 μ M and 40 nCi ml⁻¹, respectively. The protoplasts were incubated at 37 °C and six 10 μ l additions of 10 μ M-H₂O₂ were made at 2 min intervals. After a total incubation of 15 min, the protoplasts were washed six times in the stabilized buffer, lysed in a large volume of 100 mM-Tris/HCl buffer, pH 7.2, containing 10 mM-MgCl₂ and fractionated as described previously (Marriott, 1975*a*; Fig. 1). Control incubations (1 ml volume, otherwise identical conditions) without H₂O₂ and/or lactoperoxidase were performed simultaneously. Samples were removed during the fractionation procedure for determination of radioactivity.

The proportion of radioactivity precipitable by 5% (w/v) trichloroacetic acid (TCA) was determined by collecting the precipitate on membrane filters (Oxoid) and washing three times with 5 ml 1% (v/v) acetic acid. Radioactivity determinations were made using a Packard 3383 scintillation spectrometer and scintillant containing 5 g 2,5-diphenyloxazole l⁻¹ in Triton X-100/toluene (1:2, v/v). Purified plasma membrane preparations, which had been labelled from the outside, were subjected to further radioactive labelling to identify those proteins exposed on the inner membrane surface. The procedure used was similar to that described for intact protoplasts, except that a smaller (1 ml) incubation volume was used.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Polyacrylamide slab gels were constructed as follows. The separating gel was prepared from 10 ml solution A (1 M-HCl, 48 ml; Tris, 36.3 g; *N,N,N',N'*-tetramethyl-1,2-diaminoethane, 0.23 ml; H₂O to 100 ml), 13.3 ml solution B (acrylamide, 30 g; bisacrylamide, 390 mg; H₂O to 100 ml), 16.9 ml H₂O, 0.4 ml 10% (w/v) sodium dodecyl sulphate (SDS), and 0.4 ml 10% (w/v) ammonium persulphate. The stacking gel was made from 2.5 ml solution B, 1.875 ml solution C (1 M-HCl, 48 ml; Tris, 5.98 g; *N,N,N',N'*-tetramethyl-1,2-diaminoethane, 0.46 ml; H₂O to 100 ml), 10 ml H₂O, 0.15 ml 10% SDS and 0.15 ml 10% ammonium persulphate. The gels were run at a constant current of 40 mA; the electrophoresis buffer (Tris, 1.5 g; glycine, 7.2 g; H₂O to 1 l) contained 0.1% SDS and bromophenol blue as tracking dye.

Protein samples for electrophoresis were solubilized at a concentration of 1 mg ml⁻¹ in a mixture containing: 1% (w/v) SDS, 250 μ M-phenylmethylsulphonyl fluoride, 25 mM-dithiothreitol, 1 mM-EDTA, and 5% (w/v) sucrose. Samples were incubated at 45 °C for 30 min and insoluble material was removed by centrifugation. Between 100 and 200 μ g protein was applied per sample well.

After electrophoresis, the gels were fixed and the proteins were stained using the shorter procedure of Fairbanks, Steck & Wallach (1971). Glycoproteins were stained using the periodate-Schiff reagent (Fairbanks *et al.*, 1971).

Fig. 1. Discontinuous sucrose density gradient used to fractionate the 5000 g pellet from lysed protoplasts of *Candida albicans*. The gradients were made up in 3 × 1 in cellulose nitrate ultracentrifuge tubes and centrifuged for 3 h at 82 000 g (av.) in an SW27 rotor, using a Beckman L5 50 ultracentrifuge. The position of the fractions mentioned in the text is indicated.

Autoradiography. After being photographed, the stained gels were dried down under reduced pressure on to Whatman 3MM chromatography paper. They were covered with a sheet of Kodak Blue brand X-ray film and stored between glass plates until sufficient blackening of the film was observed after development in Ilford contrast FF.

Preparation of mannan-protein. Cell walls of *Candida albicans* were prepared using a Braun model MSK homogenizer (Braun, Melsungen, West Germany). After washing and lipid extraction, wall glycoproteins were extracted by the method of Falcone & Nickerson (1956). The solubilized material was extensively dialysed against distilled water before being freeze-dried. The lyophilized mannan-protein was redissolved in 12 ml distilled water and any insoluble material (putative glucan-protein) was collected by centrifugation.

Bulk plasma membrane preparation. Plasma membranes were also prepared by disrupting the cells in the Braun homogenizer. Walls were removed by centrifuging at 1000 g for 5 min and crude membranes were sedimented at 10000 g for 30 min. This preparation was then fractionated by discontinuous sucrose density gradient flotation centrifugation as above.

Mannose incorporation. The incorporation of mannose from GDP[¹⁴C]mannose (The Radiochemical Centre, Amersham) was measured using incubation mixtures which each contained (in 30 µl): 5 mM-Tris/HCl (pH 7.4); 7 mM-MgCl₂; 5 mM-MnCl₂; 0.2 mM-GDP[¹⁴C]mannose (100 nCi); and 20 to 50 µg protein. The reaction was allowed to proceed at 37 °C and stopped after suitable incubation periods by adding 0.2 ml 10% (w/v) TCA. The precipitate was collected by filtration, washed and assayed for radioactivity (Lehle & Tanner, 1974).

The ability of the membrane preparation to use exogenous acceptors was tested by incorporating mannose (20 mM) or *C. albicans* cell wall mannan-protein into the incubation mixture. When mannose was the exogenous acceptor, the reaction was stopped by adding 0.2 ml ethanol and the products were separated by thin-layer chromatography.

To determine the nature of the products of the reaction samples of the TCA precipitate were subjected to either β-elimination (24 h, 21 °C, 0.1 M-NaOH) or acid hydrolysis (1 h, 105 °C, 3 M-HCl). Following β-elimination, the reaction mixture was neutralized and dialysed. Both diffusible and non-diffusible material were assayed for radioactivity. Acid hydrolysates were evaporated to dryness over NaOH, taken up in a small volume of water and separated by thin-layer chromatography.

Incorporation into lipid-soluble material was measured by stopping the reaction with 0.5 ml chloroform/methanol (2:1, v/v). Lipid-soluble radioactivity was extracted as described by Gold & Hahn (1976). The effect of temperature on the functioning of the enzyme preparation was studied by carrying out incubations at a range of temperatures from 0 to 40 °C.

Other analytical methods. After acid hydrolysis and neutralization (Marriott, 1975a), concentrated samples of mannan-protein were applied to Whatman no. 1 chromatography paper and separated by descending chromatography using butan-1-ol/acetic acid/water (3:1:1, by vol.) as solvent (Sentandreu & Northcote, 1969). Chromatograms were stained using the Wilson dip (Wilson, 1959) and sugars were identified by comparing their *R_F* values with those of standards. Thin-layer chromatography was performed using pre-coated plastic plates (Camlabs, Cambridge) in the following solvent systems: butan-1-ol/ethyl acetate/acetic acid/water (40:30:25:40, by vol.) and ethyl acetate/pyridine/water (5:3:2, by vol.). The thin-layer plates were cut into 2 mm sections and assayed for radioactivity.

Protein was assayed by measuring the *E*₂₈₀/*E*₂₆₀ ratio.

Table 1. *Lactoperoxidase-catalysed ^{125}I iodination of intact protoplasts of *Candida albicans* and the percentage distribution of radioactivity associated with membranous material from the 5000 g pellet after osmotic lysis*

The results are the means of three experiments; standard deviations are given.

	Controls		Experimental
	No H_2O_2	No H_2O_2 or lactoperoxidase	
Washed protoplasts (TCA-precipitated c.p.m.)	$1.7 \pm 0.3 \times 10^3$	$7.4 \pm 0.5 \times 10^2$	$3.1 \pm 0.4 \times 10^5$
Incorporation of radioactivity (%)	0.0062 ± 0.0009	0.0039 ± 0.0003	0.21 ± 0.02
Fraction	Total c.p.m. (%)	TCA-precipitated c.p.m. (%)	$10^{-2} \times$ Specific activity [TCA-precipitated c.p.m. (mg protein) $^{-1}$]
Y-1	5 ± 4	7 ± 5	3.9 ± 2.0
Y-2	11 ± 2	11 ± 1	6.7 ± 3.7
Y-3	12 ± 6	13 ± 6	7.7 ± 2.4
Y-4	15 ± 10	13 ± 10	5.9 ± 0.8
Y-5	57 ± 14	57 ± 12	30 ± 1

RESULTS

Distribution of radioactivity after iodination

The distribution of ^{125}I on fractionation of labelled protoplasts from *C. albicans* is shown in Table 1. In the absence of hydrogen peroxide and/or lactoperoxidase, little, if any, labelling took place (0.0062 % and 0.0039 % compared with 0.21 %). Some 65 to 70 % of the TCA-precipitable radioactivity was sedimented at 5000 g and after fractionation of this pellet, most radioactivity (approximately 60 %) was associated with membranous material sedimenting in band Y-5 (Fig. 1). This fraction also had the highest specific activity (Table 1). Exact recoveries of protein and radioactivity were not measured due to the variation in sample sizes during fractionation, but approximate recoveries were between 60 and 80 %.

When the material from each of the five bands was run on identical discontinuous sucrose gradients, a redistribution of both protein and radioactivity took place. The amount of radioactivity associated with fractions other than Y-5 varied greatly. This was simply due to the extremely low radioactivity of the material found in these bands and the consequent large counting error. After two centrifugations, most of this radioactivity was found in the sucrose, indicating that it was originally trapped inside membranous vesicles. Only that material from band Y-5 which re-ran in position 5 did not lose its radioactivity; indeed, it showed an increase in specific activity [to 4.8×10^3 TCA-precipitable c.p.m. (mg protein) $^{-1}$]. Some 6 to 10 % of the protein in the 5000 g pellet (representing approximately 17 mg protein) was recovered in band Y-5 after two centrifugations.

Attempts to increase the specific activity of the labelled plasma membrane by modifying the experimental conditions were unsuccessful, indicating that there was only a small number of exposed sites (tyrosine residues). This is in marked contrast to the results obtained when purified plasma membranes (Y-5) were relabelled using identical experimental conditions. In this case the specific activity was increased approximately 100-fold [to 4.2×10^5 TCA-precipitable c.p.m. (mg protein) $^{-1}$]. It appears, therefore, that isolation of the plasma membrane, which exposes the inner membrane surface, greatly increases the number of proteins available for labelling.

Fig. 2. Densitometer traces of sodium dodecyl sulphate-polyacrylamide gels: (a) *Candida albicans* cell wall mannan-protein isolated by the method of Falcone & Nickerson (1956), stained for protein or carbohydrate; (b) purified plasma membranes from *C. albicans*, stained for carbohydrate; (c) autoradiogram of purified plasma membranes isolated from protoplasts which had been labelled with ^{125}I whilst intact; (d) purified plasma membranes, stained for protein; (e) autoradiogram of purified plasma membranes further labelled with ^{125}I after isolation.

Gel electrophoresis pattern and autoradiography

Figure 2(d) shows a densitometer trace of the proteins present in purified plasma membranes from *C. albicans*. A large number of major and minor bands can be observed, some of which were stained by the periodate-Schiff procedure, indicating that they are glycoproteins (Fig. 2b). Autoradiography of samples of plasma membrane iodinated on the outer surface showed that only two glycoprotein bands were labelled (Fig. 2c). However, when membranes were iodinated on both surfaces, all proteins were labelled (Fig. 2e). The two glycoproteins exposed on the outer membrane surface run in a similar position to glycoproteins extracted from the cell wall (Fig. 2a).

Characterization of mannan-protein

Attempts to isolate mannan-protein from cell walls of *C. albicans* using the 1,2-diaminoethane technique (Korn & Northcote, 1960) were unsuccessful, although this had been reported previously (Kolarova, Masler & Sikl, 1973). A fraction containing mannose and protein was isolated, but gel electrophoresis indicated that the two components were no longer covalently linked. Alkali extraction (Falcone & Nickerson, 1956) yielded a water-soluble fraction containing mainly mannose but also a faster running component, possibly a tetrose. The insoluble 'glucan-protein' (Falcone & Nickerson, 1956) had a similar composition and gave an identical gel electrophoresis profile to the soluble cell wall glycoproteins. It seems likely that this is denatured mannan-protein.

Table 2. *Effect of exogenous mannan-protein on the incorporation of mannose from GDPmannose by purified plasma membranes from Candida albicans*

The results are the mean of at least three determinations; standard deviations are given.

Experiment	$10^{-3} \times$ Specific activity [c.p.m. min ⁻¹ (mg protein) ⁻¹]	Radioactivity released by β -elimination (%)	Radioactivity extractable by chloroform/methanol (%)
Control (mem- branes only)	2.0 ± 0.2	11 ± 2	0.7 ± 0.4
Mannan-protein	2.4 ± 0.1	12 ± 3	Not tested

Mannose incorporation

During tests of 60 min duration, purified plasma membranes incorporated ¹⁴C from GDP[¹⁴C]mannose into TCA-precipitable material (glycoprotein) in a linear fashion with time. Acid hydrolysis of the product formed, followed by thin-layer chromatography, revealed that the radioactivity incorporated was entirely in the form of mannose. Approximately 10% of the radioactivity could be removed by β -elimination, conditions under which only *O*-glycosidic bonds are broken (Table 2). Less than 1% of the radioactivity could be extracted by chloroform/methanol, suggesting that little or no lipid intermediate was present in the reaction.

The addition of exogenous mannan-protein resulted in a stimulation of activity (Table 2). When mannose was the acceptor, radioactivity was detected on thin-layer chromatograms in positions corresponding to short-chain oligomannoses. However, since standards were not available for identifying these components, estimations of the percentage stimulation were thought invalid.

The effect of temperature on the activity of the enzyme preparation is shown in Fig. 3, in the form of an Arrhenius plot. A discontinuity was observed at 17 °C and also a loss of activity above 37 °C.

DISCUSSION

The isolation of pure preparations of plasma membrane from eukaryotic organisms is a difficult procedure, complicated by the presence of other, internal membranes. Radioactive labelling of the cell surface facilitates the localization and identification of this component during fractionation. The results obtained using lactoperoxidase-catalysed iodination indicate that relatively pure samples of plasma membranes can be isolated from *C. albicans* protoplasts. Fractionation on a single discontinuous sucrose gradient yields a product heavily contaminated with other membranous components. This is probably due to entrapment of membrane vesicles and a second centrifugation is required to give a pure preparation. A third passage through a sucrose gradient did not result in a further redistribution of protein or radioactivity.

The presence of radioactivity in bands other than Y-5 (plasma membrane) may be due to two factors: the presence of unreacted ¹²⁵I, and the presence of trapped plasma membrane. Both appear equally likely, since much of the associated radioactivity is released into the buffered sucrose on recentrifugation and is not TCA-precipitable, and there is also an appreciable redistribution of protein from all bands. Much of the radioactivity found in band Y-5 re-runs in position 5 after two centrifugations and the increase in specific activity indicates that purification has taken place. Gel electrophoresis of solubilized plasma membrane, iodinated on the outer surface only, shows two labelled glycoproteins, one of which barely enters the separating gel, a situation analogous to animal cells (Phillips & Morrison, 1971; Poduslo *et al.*, 1972). The anomalous behaviour of glycoproteins on gel electrophoresis prevents an accurate molecular weight determination, and each labelled component may represent more than one protein (Phillips & Morrison, 1971). The fact that all membrane

Fig. 3. Effect of temperature on the rate of incorporation of mannose from GDPmannose by purified plasma membranes from the yeast form of *Candida albicans*. The results are the means of three determinations and are plotted in the form of an Arrhenius plot.

proteins are available to the lactoperoxidase enzyme when both surfaces are exposed, but only two when the outer surface is labelled, is evidence for the asymmetric distribution of proteins across the plane of the plasma membrane of *C. albicans*.

Isolated mannan-protein from the wall of this organism gives two components on gel electrophoresis, both of which are heavily stained with the periodate-Schiff procedure. Again one component barely enters the separating gel, whilst both appear to correspond to the two exposed glycoproteins of the plasma membrane.

The structure of the cell wall mannan-protein of *C. albicans* is not known in as much detail as that from *Saccharomyces cerevisiae* (Sikl, Masler & Bauer, 1965). However, additional information can be inferred from the mannose incorporation data. A proportion of the mannose residues is linked to the protein via *O*-glycosidic linkages, whilst the remainder is not sensitive to β -elimination and is attached via *N*-glycosidic linkages. Work in this laboratory also indicates that the mannan-protein contains phosphorylated mannose residues (J. E. Cope, personal communication). It seems likely, therefore, that *C. albicans* mannan-protein resembles that of *S. cerevisiae* and is synthesized by a similar mechanism.

Previous work with *S. cerevisiae* (Sharma *et al.*, 1974) has shown that the attachment of the first mannose residues to the protein occurs via a lipid intermediate (dolichol). However, Lehle & Tanner (1974) did not find that this intermediate participated in mannan biosynthesis when crude plasma membrane preparations were used. A similar result has been obtained with *C. albicans*, which is in contrast to the results obtained studying the incorporation of mannose into growing cells. In this case 8 to 10% of the mannose was found to be lipid-soluble (Marriott, unpublished data). It appears, therefore, that those mannose residues which are attached to the protein via a lipid intermediate are added before the glycoprotein reaches the plasma membrane. However, further mannosylation reactions can occur at the plasma membrane level. From the evidence that a membrane-bound protein acceptor is involved in mannan-protein biosynthesis (Lehle & Tanner, 1974; Sharma *et al.*, 1974), it seems reasonable to suggest that there is a biosynthetic relationship between the exposed glycoproteins of the plasma membrane and the wall mannan-protein. Recent work by Horisberger, Rosset & Bauer (1976) on the localization of mannan at the surface of yeast protoplasts also supports this view.

The effect of temperature on the membrane-bound enzyme is important in two respects. Firstly, the break in the Arrhenius plot indicates that the enzyme is sensitive to its lipid environment (Wilson, Rose & Fox, 1970). Thus, altering the fatty-acid composition of the membrane may effect wall biosynthesis, evidence for which has been presented by Douglas *et al.* (1975) with *S. cerevisiae*. Secondly, Chattaway, Holmes & Barlow (1968) have shown that temperature is important in controlling the yeast-mycelial transition in *C. albicans*. Under appropriate growth conditions, at 37 °C the yeast form is induced, whilst at 40 °C the mycelial form predominates. These workers have also shown changes in wall composition associated with this transition. The sensitivity of plasma membrane-bound mannan synthetase to temperature indicates that it may play a role in dimorphism in this organism.

It has been suggested that proteins may be lost from the plasma membrane during protoplast formation and that this may influence the observed asymmetric distribution. The likely relationship between some plasma membrane and wall glycoproteins, as well as the presence of secreted enzymes, makes it difficult to define what is meant by the word 'protoplast'. I prefer to use this term rather than 'sphaeroplast', but do so simply as a working definition for the cells remaining after cell wall lysis by 'strepzymes'. Until it is possible to identify and determine which proteins belong to the plasma membrane and which to the cell wall, the above question, although valid, cannot be answered.

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