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Identification of Glycoprotein Components of α-Agglutinin, a Cell Adhesion Protein from *Saccharomyces cerevisiae*

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Several glycoproteins which inhibit the agglutinability of *Saccharomyces cerevisiae* mating type a cells were partially purified from extracts of mating type α cells. These proteins, called α-agglutinin, were labeled with ^{125}I-Bolton-Hunter reagent. The labeled α-agglutinin showed specific binding to a cells. Such specific binding approached saturation with respect to agglutinin or cells and was inhibited in the presence of excess unlabeled α-agglutinin. Nonspecific binding was similar in a and α cells, was neither saturable nor competable, and was three- to fourfold less than the specific binding to a cells at maximum tested agglutinin concentrations. The major a-specific binding species had a low electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels and had an apparent molecular weight of 155,000 by rate zonal centrifugation. Endo-*N*-acetylglucosaminidase H digestion of the purified glycoprotein complex converted the low-mobility material to four major and several minor bands which were resolved by polyacrylamide gel electrophoresis. All but two minor peptides bound specifically to a cells. Analyses of agglutinin from *mnn* mutants confirmed the deglycosylation results in suggesting that the N-linked carbohydrate portion of α-agglutinin was not necessary for activity.

Haploid yeasts of the genus *Saccharomyces* exist in one of two alternate mating types, designated **a** and α. These cells exhibit a complex mating interaction that is mediated by oligopeptide sex pheromones (reviewed in reference 21). α cells produce the peptide α-factor, which affects **a** cells; **a** cells produce **a**-factor, which affects α cells (3, 27). The effects of each of these pheromones on the opposite mating type are reciprocal and include an increase in specific adhesion to the opposite cell type (sexual agglutinability [2, 30; D. N. Radin, Ph.D. thesis, University of California, Berkeley, 1976]), induction of a transient cell cycle arrest in G1 (8, 27), and a pronounced change in cell morphology (3, 7).

Sexual agglutination provides an excellent model system for the study of eucaryotic cell interactions and their regulation at the molecular level. The agglutination reaction has been characterized in yeasts of the genus *Hansenula* and found to be the result of the interaction of a pair of complementary glycoproteins, one from each mating type (5, 23, 24). More recently, similar molecules have been isolated from *Saccharomyces kluyveri* (18) and *Saccharomyces cerevisiae* (12, 17, 26, 28, 29, 31). These studies revealed certain common elements in sexual agglutination in yeasts. In each species except *S. cerevisiae* the agglutination molecule (agglutinin) from the cells corresponding to the **a** mating type has been found to be relatively heat stable and sensitive to reducing agents and to contain a higher proportion of carbohydrate than the corresponding molecule from the opposite mating type. By contrast, the agglutinin from mating types analogous to α cells is heat labile and stable to reducing agents and contains a smaller proportion of carbohydrate (4). Most of the studies of agglutinins in *S. cerevisiae*, however, have used autoclaving to solubilize the molecules. Therefore the reported α-agglutinin glycoprotein must be heat stable (28). Previously, we reported that sexual agglutination in *S. cerevisiae* is due to pairs of interacting molecules and that the α-agglutinin from wild-type α cells is heat labile (25). In this study, we describe the purification and partial characterization of glycoproteins from α cells mediating α-agglutinin activity. We also describe a direct binding assay for this specific cell-cell adhesion activity.

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MATERIALS AND METHODS

Materials. The following reagents were obtained from Sigma Chemical Company, St. Louis, Mo.: cycloheximide, *p*-chloromercuribenzoic acid (PCMB), Trizma base, glass beads (450 to 500 μm), α-methylmannoside, hexyl agarose, DEAE-Sephadex A-25, Amberlite XAD-2, agarose-bound concanavalin A, trypsin, chymotrypsin, and Schiff reagent. The agarose-bound hydrocarbon series was from Miles-Yeda, Kiryat Weizman, Rehovat, Israel. Agarose-bound agglutinin from *Lens culinaris* was from Accurate Chemical, Westbury, N.Y. Acrylamide, bisacrylamide, tetramethylethylenediamine, electrophoresis reference proteins, and gel filtration media were purchased from Bio-Rad Laboratories, Richmond, Calif. Sodium dodecyl sulfate (SDS) came from Pierce Chemical, Rockford, Ill. Endo-*N*-acetylglucosaminidase H (endo H; purchased from Health Research, Inc., Albany, N.Y.) was pretreated with phenylmethylsulfonyl fluoride and had no detectable protease activity with Azocoll or ^{3}H-serum albumin as the substrate.

Strains and growth conditions. *S. cerevisiae* haploid strains were obtained from the Yeast Genetics Stock Center, Berkeley, Calif. Wild-type strains X2180-1A (**a**) and X2180-1B (α) were used for all routine assays and pheromone isolations. These cells were cultured in a minimal medium (YNB) containing (in grams per liter): yeast nitrogen base (Difco Laboratories, Detroit, Mich.), 2.2; ammonium sulfate, 4.5; and glucose, 20. Cells grown for agglutinin isolation were routinely cultured in a richer medium (YNBP) that was identical in composition to YNB but supplemented with peptone (1 g/liter) (Difco). The mutant strains LB1-10B

(*mnn1 MAT***a**), LB1-22D (*mnn1* MATα), LB1-3B (*mnn2 MAT***a**), LB1-16A (*mnn2 MAT*α), LB6-5D (*mnn4 MAT***a**), and LB5-10A (*mnn4 MAT*α) were cultured in YNB medium. All cells were incubated on a rotary shaker at 30°C. Cell number was determined by optical density at 660 nm in a Bausch and Lomb Spectronic 21. These readings were calibrated by hemacytometer counts.

Methods. Protein in solution was determined by the binding of Coomassie brillant blue (Bio-Rad Laboratories). Estimates based on the method of Lowry et al. (15) gave similar results with purified α-agglutinin. Carbohydrate was determined by the phenol-sulfuric acid method (6).

Polyacrylamide gel electrophoresis was a modification of that of Laemmli (13). A 4% stacking gel was poured over a 7.5% running gel (130 by 160 by 0.625 mm). Proteins present in the latter stages of purification failed to stain with Coomassie blue, but silver staining (11) did reveal bands. Carbohydrate was detected in gels by the periodic acid-Schiff (PAS) method of staining (9).

Partial purification and assay of sex pheromones. Both sex pheromones were purified by modifications of the procedures of Strazdis and Mackay (22). **a**-Factor and α-factor purification and assay are described elsewhere (20, 25).

Agglutination assay. The agglutinability of cells was measured by the assay of Terrance and Lipke as described previously (25). Samples were run in triplicate. Agglutinability was quantitatively expressed as the agglutination index (AI), the fraction of cells agglutinating under assay conditions.

Assay of α-agglutinin activity. α-Agglutinin was assayed by its ability to inhibit the agglutinability of **a** cells. Samples of fractions to be assayed were placed in assay tubes with 1 ml (2×10^7) of induced **a** cells and enough buffer A (0.1 M sodium acetate, pH 5.0, containing 10 μg of cycloheximide per ml) to bring the total volume to 2.8 ml. The tubes were incubated on a rotary shaker at 30°C for 90 min. After incubation, 0.2 ml of buffer A containing 2×10^7 cells was added to each tube, vortexed, compacted, suspended, and read as for the agglutination assay (25). At doses below 3.5 U, the effect of α-agglutinin on **a** cells was linear (25). One unit of α-agglutinin activity was defined as the amount needed to lower the AI of induced **a** cells 0.1 AI unit below the control value.

Reversal of binding. Since α-agglutinin should inhibit **a** cell agglutinability by occupying available agglutinin receptors, the inhibition should be reversible under conditions that cause dissociation of the bound agglutinin. In contrast, activities which destroy cell surface receptors would render the cells permanently nonagglutinable, providing there is no de novo synthesis of receptors. Conditions for α-agglutinin dissociation were the same as those which reverse agglutination (K. Terrance, Ph.D. thesis, City University of New York, 1983). Either high urea concentration or high pH was used to test reversibility. To dissociate bound α-agglutinin from **a** cells, the agglutinin-treated cells were suspended in sodium acetate buffer containing cycloheximide (10 μg/ml) and 8 M urea. After incubation at room temperature for 30 min, the cells were washed three times with fresh buffer without urea. They were then agglutinated and measured as before. Alternatively, the agglutinin-treated cells were incubated for 30 min in 0.05 M Tris (pH 9.0) with cycloheximide, followed by a 30-min incubation in sodium acetate (pH 5.0). There was no difference in reversibility when agglutinin-treated **a** cells were exposed to reversal reagents before or after α cell addition and agglutination. Therefore, the standard procedure was to incubate the **a** cells with the α-agglutinin, add the α cells, and assay agglutinability as described above. Control tubes (no α-agglutinin added) and tubes in which agglutination was inhibited were then incubated in Tris buffer and reassayed as described above. This procedure is called reversal. α-Agglutinin activity was considered reversible if the α-agglutinin-treated cells recovered at least 95% of the agglutinability of cells not treated with α-agglutinin.

TABLE 1. Purification of α-agglutinin

Fraction	Activity (U)	% Reversibility	Sp act (U/mg)	Vol (ml)
Crude extract	112,000	50	40	372
Ammonium sulfate	36,000	100	47	90
DEAE-Sephadex				
50 mM NaCl	2,700	100	2,100	30
100 mM NaCl	1,000	100	3,700	30
Lentil lectin-agarose[a]	3,550	100	27,000	4
Hexyl-agarose	4,000	ND[b]	ND	3

[a] 500 U of the pooled 50 and 100 mM NaCl fractions from the DEAE-Sephadex column were applied to the lentil lectin-agarose column. The active fractions from this column were applied to hexyl-agarose. Total units are extrapolated from the results of this purification.

[b] ND, Not determined.

The inclusion of cycloheximide in the agglutination and reversal buffers effectively prevents de novo synthesis of α-agglutinin receptors (25, 29; P. Lipke, unpublished data). Furthermore, **a** cells retain their original level of agglutinability not only during agglutination and reversal (25), but also during the assay and reversal with α-agglutinin (Table 1) (Terrance, Ph.D. thesis). Therefore, it appears that cell surface levels of α-agglutinin receptors remain constant during the assay and reversal procedures.

Purification of α-agglutinin. Large cultures (100 liters) of X2180-1B were grown at 30°C in YNBP medium in a Magnaferm Fermentor (New Brunswick Scientific Co., New Brunswick, N.J.). The cells were harvested at 3.4×10^8 cells per ml. To avoid heat treatment and increase yield (10), we purified α-agglutinin from broken-cell extracts. Packed cells were divided into blocks (approximately 70 g each) and stored at −20°C. For extraction, one frozen block of cells was thawed and suspended in 150 ml of buffer B (0.1 M sodium acetate [pH 5.0] containing 0.03% Triton X-100 and 1 μM PCMB). The inclusion of detergent and protease inhibitor was found to stabilize agglutinin activity. The cell suspension was transferred to the chamber of a Bead Beater blender (Biospec Products, Bartelsville, Okla.), and the remaining space in the chamber was filled with glass beads. The chamber was submerged in an ice bath, and the cells were homogenized four times for 1.5 min each, with 1-min cooling periods between homogenizations. This treatment was usually sufficient to disrupt 90% of the cells, as judged by phase contrast microscopy.

The resulting extract was cleared by centrifugation twice at 28,000 × *g* for 10 min. The pellet was discarded, and the supernatant was maintained at 4°C and brought to 50% saturation with crystalline ammonium sulfate. This mixture was then cleared by centrifugation at 28,000 × *g* for 10 min. The pellet was discarded, and the supernatant was brought to 100% saturation with ammonium sulfate. This mixture was centrifuged as above, the supernatant was discarded, and the pellet was suspended in extraction buffer and dialyzed against 4 liters of extraction buffer. Dialysis was carried out at 4°C over a period of 36 to 48 h with at least two changes of buffer. The dialysate was centrifuged and applied to a column (1.8 by 6.5 cm) of DEAE-Sephadex A-25 (4°C)

previously equilibrated with buffer B. The column was washed with 20 ml of buffer B and then eluted in a stepwise manner with 20 ml of buffer containing 50, 100, or 500 mM NaCl. Two-milliliter fractions were collected.

Fractions from the 50 and 100 mM NaCl elutions were found to have α-agglutinin activity that was fully reversible (see above and Table 1). Such fractions were pooled, brought to 1 mM in $CaCl_2$ and $MnCl_2$, and applied to a column (1.2 by 0.9 cm) of agarose-bound lectin from *Lens culinaris* (lentil lectin) at room temperature. The column was washed with 10 ml of buffer B with 1 mM Ca^{2+} and Mn^{2+} added and then eluted successively with 10 ml of the same buffer containing 0.1 M NaCl and then 0.1 M NaCl plus 1 M α-methylmannoside. Active and reversible fractions from the α-methylmannoside elution were pooled and dialyzed (4°C) against 0.01 M sodium acetate (pH 5.0) with 0.003% Triton X-100 and 1 μM PCMB (buffer C). This dialysate was applied to a column (1.2 by 0.9 cm) of agarose-bound hexane previously equilibrated in the same buffer at room temperature. The column was washed with 3 ml of buffer and then eluted with buffer C containing NaCl at 100, 200, 500, and 1,000 mM. Activity eluted with 100 mM NaCl. α-Agglutinin was not retained on small columns of native agarose or of butane-substituted agarose. Purified α-agglutinin was lyophilized from buffer C with 50 mM mannitol added and stored at 4°C.

Table 1 shows a purification that was 570-fold over the ammonium sulfate fraction with a 10% yield. Such figures were typical, with final specific activities ranging from 12,000 to 27,000 U/mg for nine preparations. Two preparations assayed after hexyl-agarose purification had activities of 20,000 and 22,000 U/mg.

Purification of α-agglutinin with **a** cells as an affinity reagent was unsuccessful. Although α-agglutinin bound to the cells, only small quantities could be eluted under nondenaturing conditions. No additional agglutinin was recovered after elution at high or low pH or in the presence of urea, although such treatments were necessary and sufficient for the full recovery of agglutinability of α-agglutinin-treated **a** cells. These results were consistent with the observation of binding in two states: a weak interaction which allowed elution by washing, and a strong interaction that was reversible only by denaturing reagents (14). In control experiments, α-agglutinin activity was not recovered following treatment with such denaturing reagents. The amount bound in the weak state appeared to be a minority of the activity under conditions of **a** cell excess, consistent with the results of affinity purification attempts (14).

Isotopic labeling and binding assays. α-Agglutinin purified through the lentil lectin step was dialyzed and lyophilized and redissolved in 0.2 M sodium phosphate buffer, pH 8.8. A 200-μg amount of protein was reacted with 0.3 mCi of ^{125}I-Bolton-Hunter reagent (New England Nuclear Corp.) for 1 h at 0°C and overnight at 4°C. The reaction mixture was diluted 20-fold into buffer A containing 1 mM $CaCl_2$ and 1 mM $MnSO_4$ and applied to a lentil lectin-agarose column, which was washed and eluted as described above. The labeled material was stored at 4°C in the eluting buffer supplemented with 10% glycerol and 1 mg of bovine serum albumin (Sigma) per ml. Four preparations had specific radioactivities between 8×10^4 and 2.5×10^5 cpm/μg of protein.

To determine binding, we incubated labeled α-agglutinin with 2×10^6 to 5×10^7 cells in a 0.5-ml total volume. The buffer was buffer B supplemented with bovine serum albumin (1 mg/ml) and cycloheximide (10 μg/ml). Unless otherwise stated, incubation was at 30°C for 90 min. The cells were then pelleted, the supernatant solutions were aspirated, and the cells were washed twice in 0.5 ml of buffer, each wash including a 20-min incubation of cells with buffer. The washing procedure greatly reduced nonspecific binding, measured as binding to α cells, but also removed some of the specifically bound α-agglutinin. However, similar peptides are removed by washing as remain bound to the **a** cells, so washing did not alter the classes of peptides bound. The accompanying paper details the effects of the washing (14). Samples were counted in a Packard Autogamma 5650. Autoradiography was carried out on dried gels at −70°C with Kodak XAR5 film backed by DuPont Cronex Lightning-Plus screens.

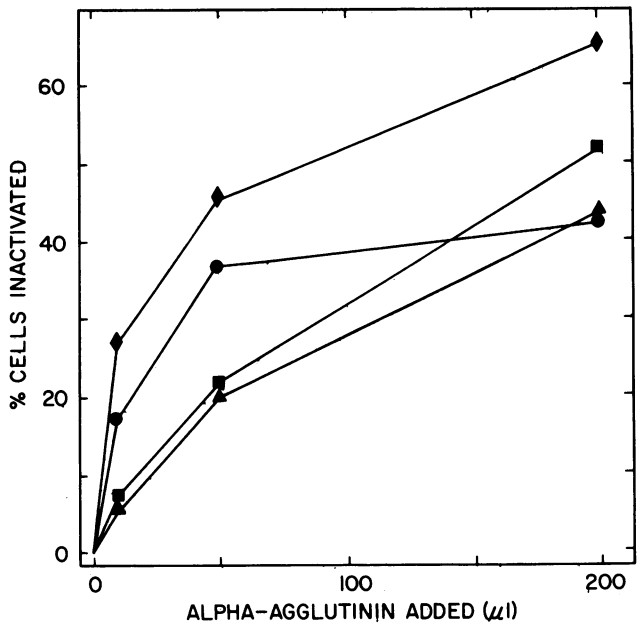

FIG. 1. Effect of α-agglutinin on **a** cells agglutinated in various combinations. α-Agglutinin purified through lentil lectin was incubated with uninduced or induced **a** cells, which were agglutinated with induced or uninduced α cells. The four agglutinable combinations that resulted were (◆) neither cell type induced, (●) α cells only induced, (■) **a** cells only induced, and (▲) both cell types induced.

RESULTS

Properties of α-agglutinin. Purified agglutinin was specific in its inhibition of **a** cell agglutinability. **a** cells were inhibited regardless of their state of pheromonal induction or the state of induction of the α cells with which they were agglutinated (Fig. 1). A given amount of α-agglutinin inactivated a larger proportion of the treated **a** cells when the cells had not been induced by pheromone. Nevertheless, high concentrations of α-agglutinin completely inactivated both uninduced and induced **a** cells (data not shown). A comparison of the initial slopes of the titration curves in Fig. 1 indicates that uninduced **a** cells were about 2.5 times more sensitive to inactivation by α-agglutinin than were α-factor-treated **a** cells. The observation implies that the number of α-agglutinin receptors was increased about 2.5-fold by the α-factor treatment. Such a result is consistent with the observed twofold increase in cellular agglutinability caused by α-factor treatment of X2180-1A (25).

TABLE 2. Sensitivity of α-agglutinin activity to carbohydrate- and protein-specific reagents[a]

Reagent	Amount	Activity remaining (%)
None		100
Endo H[b]	5×10^{-4} U	107
α-Mannosidase[b] (jack bean)	1.3 U	120
$NaIO_4$[b]	1 μmol	102
Trypsin[c]	1 μg	93
Chymotrypsin[c]	1 μg	63
Pronase[c]	1 μg	38

[a] The indicated amount of reagent was incubated with 100 U of DEAE-purified α-agglutinin containing 50 μg of carbohydrate and 45 μg of protein. Incubation was for 30 min at 25°C, except for $NaIO_4$, which reacted at 0°C in the dark. After 30 min, 1 mM phenylmethylsulfonyl fluoride was added to all enzyme digests and 1 drop of glycerol to the $NaIO_4$ reaction. Activity remaining was determined relative to a sham digestion without added reagents. In digests without added α-agglutinin there was no effect of the reagents on agglutinability of the tester cells.

[b] Digestion in 0.1 M sodium acetate buffer, pH 5.0, containing 0.03% Triton X-100.

[c] Digestion in 0.1 M Tris chloride buffer, pH 7.4, containing 0.03% Triton X-100.

Other properties of purified α-agglutinin were essentially identical to those of the protein found in crude preparations. These included pH range for activity of 5 to 7, pH range for stability of 3 to 9, and half-time of inactivation of a cells of 20 min. Purified agglutinin was active under conditions in which binding of intact a cells to α cells occurred. Activity was inhibited under the same conditions that inhibited cell-to-cell binding (25): binding at 0°C, in 5 M NaCl, at a pH below 4 or above 7, in 0.1% SDS, and in 8 M urea. Agglutinin activity was reduced by 80% in a 5-min incubation at 100°C. Agglutinin activity was sensitive to pronase and chymotrypsin, but appeared to be stable in the presence of trypsin, periodate, α-mannosidase, and endo H (Table 2).

Silver-stained polyacrylamide gels of the purified agglutinin revealed diffuse staining in several regions (Fig. 2). The area of the gels corresponding to molecular weights above 100,000 appeared darker than the higher-mobility regions. The only obviously stained band was at M_r 62,000. PAS-stained gels showed carbohydrate in the region of M_r > 125,000. The presence of substantial amounts of carbohydrate implied that the observed molecular weights were not accurate estimates (9).

When lentil lectin-purified α-agglutinin was labeled with ^{125}I-Bolton-Hunter reagent, the major labeled species was material which remained near the top of the gel. This material stained poorly with silver (Fig. 2) and not at all with Coomassie blue, but appeared highly reactive with the PAS reagent. Integration of densitometer scans of autoradiograms revealed that at least 80% of the counts were in the low-mobility material. The remainder of the radioactivity was distributed in several bands (visible in Fig. 5). However, the silver-stained material which had an apparent molecular weight of 62,000 was not labeled. Subsequent purification of the labeled material on hexyl-agarose yielded a specific binding fraction which coeluted with α-agglutinin activity and contained primarily the material which had low mobility in the gels. Minor bands at M_r 47,000, 50,000, and 53,000 were removed by this purification step.

Binding of ^{125}I-α-agglutinin and demonstration of binding species. ^{125}I-α-agglutinin bound to both a and α cells. The binding to α cells appeared to be nonspecific in that binding was a linear function of agglutinin added and cells added (Fig. 3). Such binding was not saturable at the highest

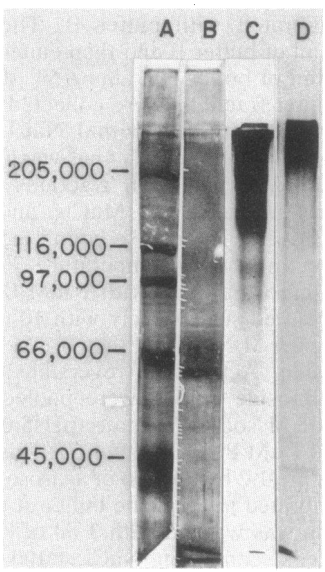

FIG. 2. SDS-polyacrylamide gels of purified α-agglutinin. Lanes: A, molecular weight standards, silver stained, 0.3 μg of each protein added: B, lentil lectin-purified α-agglutinin, silver stained, 16 μg of protein added: C, lentil lectin-purified α-agglutinin stained with PAS reagent; D, autoradiogram of ^{125}I-α-agglutinin purified through the lentil lectin step.

concentrations tested (6 μg of α-agglutinin per ml, 10^9 cells/ml). The binding to α cells was typically about 4% of the added counts and could be reduced to 1% or less by washing the cells after binding. The amount of agglutinin bound to α cells was not altered by the presence of excess unlabeled α-agglutinin. Binding of ^{125}I-α-agglutinin to the a/α diploid strain X2180 was similar to binding to the α strain X2180-1B (data not shown).

^{125}I-α-agglutinin bound to a cells in three modes. Nonsaturable binding was similar to α-agglutinin binding to α cells and was assumed to be nonspecific. In addition, there

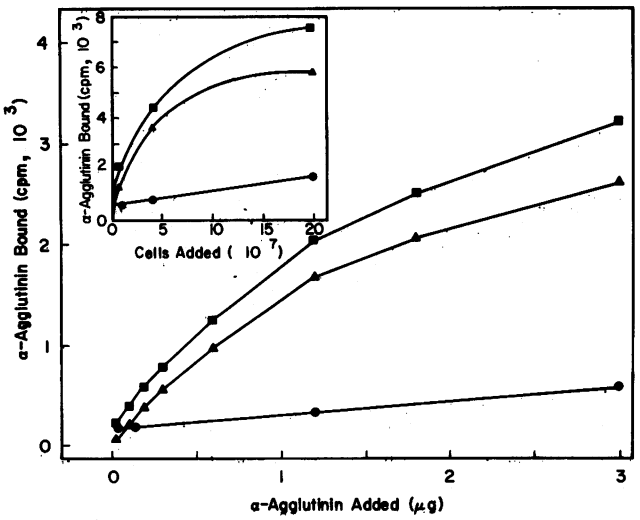

FIG. 3. Binding of ^{125}I-agglutinin to haploid cells. Radioactive agglutinin was incubated with 10^7 a or α cells as described in Materials and Methods, and the cells were washed twice before counting. Symbols: ■, binding to a cells; ●, binding to α cells; ▲, difference between a and α cells. Inset: Binding of 1.3 μg of ^{125}I-α-agglutinin to cells. Symbols: same as the main panel.

were at least two modes of **a** cell-specific binding. **a** cells incubated with α-agglutinin bound 5 to 17% of the added label in addition to the nonspecific binding, depending on the preparation of labeled agglutinin. The additional binding appeared to approach saturation with excess cells or α-agglutinin and could be competed with by unlabeled α-agglutinin (Fig. 3 and 4). Washing the cells before counting reduced the α-specific binding by about half in the presence of excess agglutinin, but reduced binding only 3 to 10% in the presence of excess **a** cells. These characteristics are expected if the ^{125}I-α-agglutinin binds specifically to the **a** cells in weak and tight modes. Such a model is discussed in the accompanying article (14).

The characteristics of the specific binding of ^{125}I-α-agglutinin to **a** cells were similar to those of α-agglutinin inhibition of **a** cell agglutinability. Both processes occurred at similar pH ranges, were reversible by urea or pH 9 incubation under conditions which preserve cell surface agglutinin concentrations (25, 29), and occurred with apparent first-order kinetics, with half-time to equilibrium of ca. 20 min at 30°C (14). Activity in both assays was reduced by binding at 0°C (14, 25). α-agglutinin activity and ^{125}I-α-agglutinin binding to **a** cells were inhibited by preincubation of the α-agglutinin with partially purified **a**-agglutinin (P. Zhang and P. Lipke, unpublished data). Furthermore, titrations of untreated **a** cells showed similar binding characteristics, except that binding-site numbers were reduced two- to threefold. Thus, the amount of ^{125}I-α-agglutinin binding to uninduced **a** cells reflected the relative constitutive agglutinability of the X2180-1A cells (25) as well as the relative susceptibility to α-agglutinin-mediated masking of agglutination receptors (Fig. 1).

Since the majority of the labeled material did not bind to either α or **a** cells, we felt that it was essential to determine whether the binding species were representative of the preparation as a whole. Labeled α-agglutinin was incubated with cells, and the bound protein was eluted in SDS gel sample buffer at 90°C. Electrophoresis revealed that the low-mobility material and the M_r-50,000 band bound preferentially to **a** cells (Fig. 5, lanes A and B). In addition, minor bands at M_r 47,000 and 53,000 appeared to bind specifically

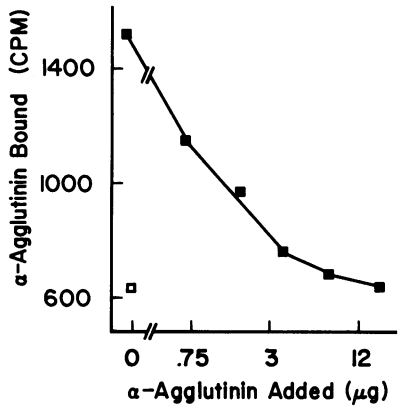

FIG. 4. Competition of unlabeled α-agglutinin with ^{125}I-α-agglutinin. Cells (2×10^7) were incubated with 0.3 μg of ^{125}I-α-agglutinin. The amount of unlabeled agglutinin indicated on the abscissa was added simultaneously with the labeled α-agglutinin. To assay binding with both weak and tight modes (14), the cells were not washed before counting. Symbols: ■, binding to **a** cells; □, binding to α cells. The binding to α cells was not affected by unlabeled α-agglutinin.

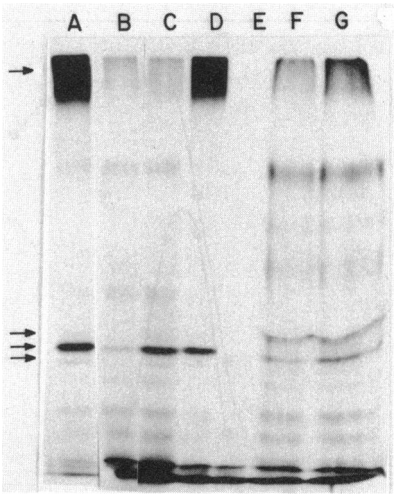

FIG. 5. Binding of ^{125}I-α-agglutinin to cells. For each lane, 1.4 μg of labeled α-agglutinin was incubated with 10^8 cells, and the bound material was extracted, electrophoresed in SDS, and autoradiographed. Arrows mark bands showing **a** cell-specific binding: from the top, low-mobility and M_r 53,000, 50,000, and 47,000, respectively. Lanes: A, material bound to **a** cells; B, material bound to α cells; C, material bound to **a** cells in the presence of 40 μg of unlabeled α-agglutinin; D, material bound to **a** cells after two washes in buffer; E, material bound to α cells after two washes in buffer; F and G, supernatant (unbound material) from incubations with **a** cells (F) and α cells (G).

to **a** cells. The relative amount of material in the M_r 47,000, 50,000 and 53,000 peptides was highly variable in different preparations (compare Fig. 2, 5, and 6). The preferential binding of the four peptides labeled in Fig. 5 was even clearer when the cells were washed before extraction of the

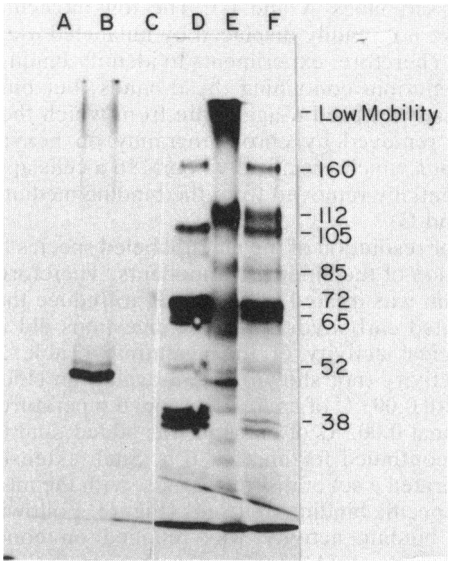

FIG. 6. Binding of deglycosylated ^{125}I-α-agglutinin. Samples (45 μg) of endo H-digested or agglutinin incubated without added enzyme were incubated with 5×10^7 **a** or α cells, the cells were washed in incubation buffer, and the bound agglutinins were extracted and separated. Lanes: undigested α-agglutinin bound to α cells (A) or **a** cells (B); endo H-digested α-agglutinin bound to α cells (C) or **a** cells (D); α-agglutinin undigested (E) and endo H digested (F). Numbers indicate 10^3 M_r.

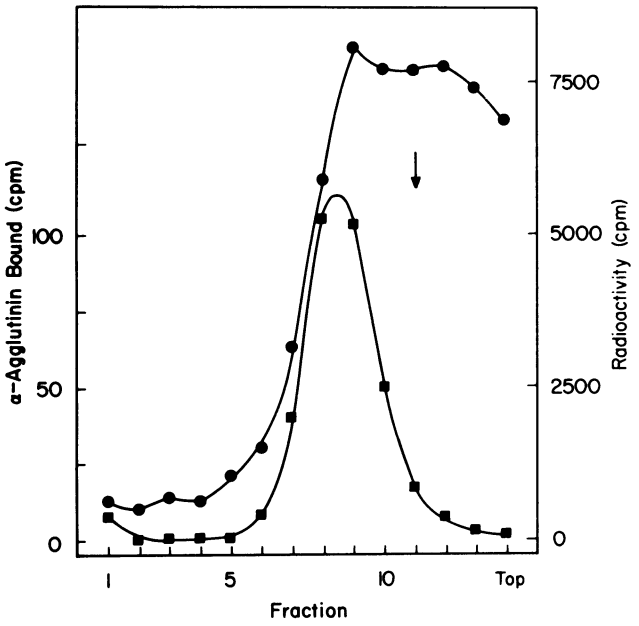

FIG. 7. Rate zonal centrifugation of α-agglutinin. ^{125}I-labeled α-agglutinin was sedimented for 15 h at 26,000 rpm in an SW-40 rotor. Fractions (0.5 ml) were taken, and 50-μl samples were assayed for binding to **a** and α cells. The difference in binding to the two cell types is plotted (■), as well as radioactivity in each fraction (●). The arrow marks the position of bovine serum albumin (4.7s, 66,000 daltons).

labeled peptides (Fig. 5, lanes D and E). Densitometry of lane D revealed that at least 80% of the bound counts were in the low-mobility material. The intensities of all four bands were reduced when the incubation included unlabeled α-agglutinin (cf. lanes A and C). The low-molecular-weight bands were not readily displaced by unlabeled α-agglutinin, however. Therefore, experiments to identify binding species used α-agglutinin containing these bands, but binding and activity analyses used α-agglutinin from which these bands had been removed by chromatography on hexyl-agarose. Those bands which specifically bound to **a** cells appeared to be preferentially removed from the binding medium (Fig. 5, lanes F and G).

The poor resolution of the major labeled species hampered identification of the binding components. Therefore, labeled α-agglutinin was treated with endo H to reduce the amount of associated carbohydrate. Short digestions did not affect the biological activity of the agglutinin (Table 2) or the binding activity (not shown). ^{125}I-α-agglutinin (150 μg) was treated with 0.003 U of endo H at room temperature for 17 h, an additional 0.002 U of enzyme was added, and the digestion was continued for another 6 h. Such extensive digestions generated a set of discrete bands, with the major bands showing specific binding to **a** cells (Fig. 6). Following digestion, the binding activity was retained on concanavalin A-agarose and could be eluted with α-methylmannoside, implying that the active components contained some endo H-resistant carbohydrate. The digestion did not affect binding or biological activity of the agglutinin. Time courses of the digestion demonstrated that the pattern shown in Fig. 6, lane F, was the terminal digestion pattern (not shown). A similar incubation was carried out without endo H. The treated agglutinin was incubated with cells, the cells were washed with binding buffer, and the bound agglutinin was extracted and separated on gels. An autoradiogram was extensively exposed to demonstrate the minor bands (Fig. 6). Densitometry of the autoradiograms revealed that the bands at M_r 65,000, 72,000, 105,000, and 160,000 represented over 80% of the counts in lane F (see also Fig. 8 of reference 12). Less dense bands had apparent molecular weights of 37,000, 38,000, 39,000, 52,000, 85,000, and 112,000. Of these species, all but the M_r 85,000 and 112,000 bands bound preferentially to **a** cells (Fig. 6, lanes C and D). The three bands of lowest molecular weight bound **a** cells avidly, but were not present in digests of hexyl-agarose-purified α-agglutinin. This observation suggests that these bands were products of digestion of the M_r 47,000, 50,000, and 53,000 binding components, which were eliminated by such purifications. Incubation without endo H did not appear to affect the binding preference for **a** cells, although the amount of active low-mobility material appeared to be much reduced during the mock digestion (lanes A, B, and E). The result suggests that the low-molecular-weight forms of α-agglutinin may be proteolytic fragments of the low-mobility forms.

Estimation of molecular weight. α-Agglutinin purified through lentil lectin-agarose was adsorbed to several agarose and acrylamide gel filtration media, so no estimate of molecular weight could be obtained by gel filtration. As an alternate strategy, the agglutinin was subjected to rate zonal centrifugation (16). The major agglutinin peak had a sedimentation value of 7.8s (Fig. 7). Assuming a partial specific volume of 0.725 ml/g (16), the sedimentation coefficient corresponds to a molecular weight of 155,000. The sedimentation coefficients and molecular weight estimates were similar with either bovine serum albumin or rabbit muscle aldolase as the standard and were relatively insensitive to differences in partial specific volume within the range observed for glycoproteins (16). The sedimentation values were not detectably affected by the presence of 0.03% Triton X-100. For six runs of three different preparations of α-agglutinin, the major peak had an apparent molecular weight of 155,000 ± 5,000 (standard error). A minor peak (6.7s, M_r 128,000 ± 2,000) was seen in three runs. Two centrifugations of endo H-treated ^{125}I-α-agglutinin yielded molecular weight estimates of 78,000 and 81,000 for the peak fractions, implying that about half of the mass of the α-agglutinin is endo H-susceptible carbohydrate. The major binding species, shown at M_r 65,000 and 72,000 in Fig. 6, were the major constituents of these fractions (not shown).

Agglutination in *mnn* strains. There exist a number of mutant strains of *S. cerevisiae* with well-characterized alterations in the structure of mannan, the bulk glycoprotein of the cell wall. Mutants carrying mutations in the locus *MNN1* have shortened mannose side chains, *mnn2* mutants lack

TABLE 3. Agglutination of *mnn* strains

a cells	α cells	Agglutination (% of wild type)	
		Control **a** cells	Induced **a** cells
Wild type	Wild type	100	100
mnn1	Wild type	29	81
Wild type	*mnn1*	130	103
mnn2	Wild type	129	100
Wild type	*mnn2*	151	101
mnn2	*mnn2*	133	109
mnn4	Wild type	56	103
Wild type	*mnn4*	113	99
mnn4	*mnn4*	69	103

distal side chains, and *mnn4* strains do not have phosphodiesters (1). We tested these mutants for agglutinability and found that they all showed specific **a** to α agglutination, even with wild-type cells (Table 3). These results confirm those previously reported by Radin (Ph.D. thesis). We used titration of agglutinated cells with disruptive reagents to try to distinguish between different types of agglutination. Such titrations are sensitive indicators of the resistance of intercellular bonds to disruptions. When *mnn2* cells were agglutinated in all combinations with wild-type testers in the presence of different concentrations of urea, the results were indistinguishable from those with the wild-type cells (Fig. 8). The results of such titrations were similar regardless of whether the **a** cells had been pretreated with pheromone. Therefore, the adhesions between *mnn2* cells and wild-type cells were as resistant to urea as were those between wild-type cells. By this functional criterion, the agglutinins of the mannan biosynthesis mutants were not different.

DISCUSSION

The identification of α-agglutinin peptides depends on the specific binding of ^{125}I-α-agglutinin to **a** cells representing the same interaction that is assayed by agglutination inhibition. The concordance of the results in the agglutination and binding assays strongly argues that they determine the same interactions. Both assays show similar mating type specificity, kinetics, and effects of washing after binding (14, 25). Titration of cells with α-agglutinin before and after pheromone induction showed similar increases in receptor numbers in both assays, and the results are in agreement with the observed level of constitutive agglutinability of these strains (25). Finally, studies with several strains with lower agglutinability than X2180-1A show comparable receptor concentrations in cellular agglutination assays and ^{125}I-α-agglutinin binding assays (B. Soden and P. Lipke, unpublished observations).

Deglycosylation of α-agglutinin removed about half the mass and revealed that there are several peptides associated with the active agglutinin. We have not determined whether the various peptides are alternate forms of the agglutinin derived from proteolysis or processing differences or subunits of an active complex or are due to aggregation of inactive peptides with active ones. The four major deglycosylated peptides of M_r 160,000, 105,000, 72,000, and 65,000 together account for at least 80% of the binding species. In addition, most of the minor peptides also bind preferentially to **a** cells. Since we have not separated these peptides, it is not clear whether one or more of them are necessary for inhibition of cellular agglutinability. However, the accompanying paper (14) shows that in an endo H-treated preparation of hexyl-agarose-purified α-agglutinin, the M_r 160,000, 105,000, and 72,000 peptides bound specifically and saturably to **a** cells. All specific binding species had a single apparent association constant. Therefore, it is likely that these three species are components of an active complex of peptides or are related peptides with common binding characteristics.

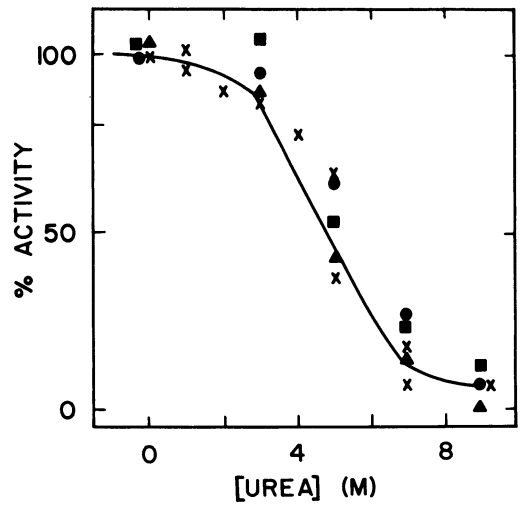

FIG. 8. Urea titration of agglutinability in wild-type cells and *mnn2* mutants. Cells were mixed as indicated and agglutinated in the presence of various concentrations of urea in agglutination buffer. Activity was determined as the percentage of maximal agglutination for each agglutinating pair. Symbols: ×, wild-type **a** cells agglutinated with wild-type α cells; ●, *mnn2* **a** cells agglutinated with *mnn2* α cells; ▲, *mnn2* **a** cells agglutinated with wild-type α cells; ■, wild-type **a** cells agglutinated with *mnn2* α cells.

In some preparations, there were several peptides whose association with α-agglutinin activity was not clear. There was one silver-stained band (M_r 62,000) which was not labeled with Bolton-Hunter reagent and therefore was not detected in autoradiographs. Moreover, this material was not detectable on silver-stained gels of deglycosylated α-agglutinin. Therefore, we cannot tell whether this peptide is a component of the active material. We suspect that this material is a contaminant, since it was present in various amounts in different preparations of α-agglutinin.

Different preparations appeared to contain different amounts of the M_r 47,000, 50,000, and 53,000 peptides (compare Fig. 2, 5, and 6 with Fig. 5 and 6 of reference 14). When these peptides were removed from the preparations by chromatography on hexyl-agarose, there was no significant loss of activity (Table 1). Therefore, these peptides cannot be essential for α-agglutinin activity. Nevertheless, these bands bind avidly to **a** cells and would be expected to contribute to activity and **a** cell binding when present in high concentrations. The variability in abundance, together with the apparent enrichment of the M_r 50,000 bands in the sham digest (Fig. 6 lane B), imply that the lower-molecular-weight bands may be biologically active degradation products of the α-agglutinin.

A purified α-agglutinin from *S. cerevisiae* has been reported previously as a glycoprotein of 1.3×10^6 daltons (28). A form solubilized from broken cells was reported to have a molecular weight of 200,000 by gel filtration (28). The size of peptides following deglycosylation was not reported. These investigators solubilized agglutinin by autoclaving cells and also used boiled tester cells (100°C, 5 min) to measure agglutinin activity in the agglutination assay. While the α-agglutinin we have purified was more heat stable than the extracts investigated earlier (25), the majority of the activity would be lost during autoclaving. This is in agreement with the general observation, made for several species and two genera, of the heat lability of the agglutinin isolated from the mating type analogous to α (4).

That α-agglutinin can be partially deglycosylated without loss of activity implies that N-linked carbohydrate is not essential. The result confirms the observation that **a**-factor induction of α-agglutinin is not inhibited by concentrations of tunicamycin that inhibit glycosylation (21; Terrance, Ph.D. thesis). Similarly, the agglutinability of the *mnn* strains argues against an essential role for N-linked carbohydrate in the function of α-agglutinin.

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