Comparison of Dose-Response Curves for $\alpha$ Factor-induced Cell Division Arrest, Agglutination, and Projection Formation of Yeast Cells

IMPLICATION FOR THE MECHANISM OF $\alpha$ FACTOR ACTION*

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$\text{MAT}^{a}$ cells of the yeast Saccharomyces cerevisiae produce a polypeptide mating pheromone, $\alpha$ factor. $\text{MAT}^{a}$ cells respond to the pheromone by undergoing several inducible responses: the arrest of cell division, the production of a cell surface agglutinin, and the formation of one or more projections on the cell surface commonly termed the "shmoo" morphology. Dose-response curves were determined for each of these inducible responses as a function of $\alpha$ factor concentration. It is shown that under conditions commonly employed in previous studies, the dose-response for cell division arrest is determined by the rate at which cells inactivate the $\alpha$ factor. In order to achieve conditions where inactivation would not be the dominant parameter, the cell division response to $\alpha$ factor was monitored at low cell densities. Under conditions of essentially no $\alpha$ factor destruction, the dose of $\alpha$ factor at which cells exhibit a half-maximal response for cell division arrest ($2.5 \times 10^{-10} \text{ M}$) is nearly the same as that at which cells exhibit a half-maximal response for agglutination induction ($1.0 \times 10^{-10} \text{ M}$). On the contrary, the half-maximal response for projection formation was obtained at doses of $\alpha$ factor 2 orders of magnitude higher ($1.4 \times 10^{-8} \text{ M}$). These results are consistent with the same high affinity $\alpha$ factor receptor mediating both cell division arrest and agglutination induction. A different system of lower affinity must mediate projection formation. Alternatively, if the same system and receptor are used, then a much higher occupancy is required for the induction of projections compared to division arrest and agglutination induction.

Polypeptide pheromones are secreted into the medium by each of the two haploid mating types of Saccharomyces cerevisiae yeast cells, a-factor by $\text{MAT}^{a}$ cells (1) and $\alpha$ factor by $\text{MAT}^{a}$ cells (2). The purpose of these pheromones is to bring about the initial stages of conjugation (for recent reviews see Refs. 3-6).

$\alpha$ factor has been purified (7) and sequenced (8, 9). A factor causes $\text{MAT}^{a}$ cells to arrest cell division at the highly significant "start" step which has been characterized genetically (10, 11). Cells arrest at start as unbudded mononucleated cells prior to DNA synthesis (12). Cells grow up to 30 times their normal size when arrested (13). Arrested cells eventually recover to resume cell division; longer arrest times (i.e. later recovery) occur at higher $\alpha$ factor concentrations (14). Recovery from division arrest has been attributed to the inactivation of $\alpha$ factor by a cell-associated protease which cleaves a factor between Leu-6 and Lys-7 (15-17). Consistent with this are the independently reported observations that faster $\alpha$ factor inactivation (16) and earlier recovery from arrest (17) both occur at higher cell densities.

$\alpha$ factor has many other effects on the cell. It induces the appearance of an agglutinin on the surface of $\text{MAT}^{a}$ cells which mediates agglutination with $\text{MAT}^{a}$ cells. Agglutination aggregates are formed of 100-500 cells with an $\alpha a$ ratio of approximately 1 (18, 19). $\alpha$ factor also induces a characteristic morphological change in $\text{MAT}^{a}$ cells called the "shmoo" (20). Shmoo cells typically have one or two narrow projections extending from the cell body. The function of the projection appears to be that of a copulation tube since cell fusion occurs at the tip of the projection (20, 21). Biochemical changes in the cell wall of the projection compared to the remainder of the cell or untreated cells (22, 23) presumably mediate fusion at the projection tip during conjugation.

In addition to the physiological effects mentioned above, $\alpha$ factor induces intracellular protein degradation and vacuole permeability (24), alters the molecular weight of a specific phosphoprotein in $\text{MAT}^{a}$ but not $\text{MAT}^{a}$ cells (25), and at high concentrations inhibits adenylate cyclase in isolated yeast cell membranes (26). $\alpha$ factor induces the formation of a folded chromosome structure which is distinctly different from stationary phase G0 and exponential phase G1 structures (27). It has been concluded that $\alpha$ factor induces a microdifferentiated pathway in the cell (27).

In sorting out this plethora of responses to $\alpha$ factor, we would like to know the sequence of events which are necessary and sufficient for each response and what common elements are used in the mechanisms of the various responses. A quantitative kinetic approach to this problem is described here, in which dose-response curves for various changes induced by $\alpha$ factor are compared under an identical set of conditions. This type of quantitative comparison can help to elucidate whether the different responses are mediated through a common element, and in fact the data are consistent with arrest and agglutinin induction occurring through a common $\alpha$ factor saturable site, or receptor.

Several assays for $\alpha$ factor have been reported but there are difficulties associated with them. The most common involves a measure of shmoos (i.e. unbudded cells containing

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projections) versus α factor concentration (7, 16). The shmoo cell assays are semiquantitative (3), and the average pure α factor concentration which I estimate to produce a half-maximal response has varied from $2 \times 10^{-14}$ M (9, 28, 29) to $2 \times 10^{-12}$ M (16), with a variety of intermediate values (4, 7, 17, 30). No dose-response curves of this assay have been shown in the literature. Similarly the average α factor concentration to produce a half-maximal agglutination response has varied from $6 \times 10^{-12}$ M (31) to $4 \times 10^{-6}$ M (32), with a variety of intermediate values (4, 33, 34). There is only one reported dose-response curve for cell division arrest, which is based on α factor inhibition of DNA synthesis (31). It shows an α factor concentration to produce a half-maximal response of $10^{-6}$ to $10^{-7}$ M (31, 33). These large variations in the amount of α factor to produce half-maximal responses may be related to strain and other assay condition differences, but much of the discrepancy is almost certainly due to uncontrolled α factor destruction by the MATα cell-associated protease, as will be demonstrated in this paper. It was felt that there was a need to make the assays more rigorous so that they could be quantitatively compared, and this was done for agglutination and projection formation. Two new cell division arrest assays were devised here and are the N_i/N_o and the %UB_h assays.

MATERIAL AND METHODS^1

RESULTS

Cell Division Arrest Assays—Two quantitative assays for the induction of cell division arrest by a factor were developed in this study. The first is based on the kinetics of cell number increase over time in the presence of α factor (Fig. 5A). This kinetics has been described previously (14). The cell number at 4 h (where the cells have doubled at least once) divided by the initial cell number (N_i/N_o) is plotted against initial α factor concentrations in Fig. 1A to provide a quantitative assay for α factor-induced cell division arrest at high cell concentrations (≥10^6 cells/ml).

The second assay procedure, used at both high and low cell concentrations (10^-10^6 cells/ml), is based on the kinetics of the increase in per cent unbudded cells over time after the addition of α factor (Fig. 5B). These kinetics have also been previously described (3, 14). The assay based on these kinetics is a measure of the per cent unbudded cells at 3 h (%UB_3) after the addition of α factor (Fig. 1B). The K_50 is defined as the α factor concentration which produces 50% of the maximum response in a given assay. The value of 1/K_50 is given by the x axis intercept of the double reciprocal plots. The K_50 of the N_i/N_o assay was compared to that for the %UB_3 assay at 6 × 10^5 cells/ml and found to be identical under identical conditions (see Fig. 2). This is expected because upon recovery from 100% arrest, cell number begins to change about 1 h after the %UB (14). Thus, cell number measurements are made 1 h later than %UB in order to obtain proportional changes in the two parameters at identical α factor concentrations.

Effect of Cell Concentration and α Factor Inactivation on K_50 for Cell Division Arrest—The K_50 for cell division arrest by α factor was found to depend on cell concentration above 10^6 cells/ml, but not below 10^5 cells/ml (Fig. 2).

It is likely that the dependence of K_50 on cell concentration is due to the cell-associated inactivation of α factor by proteolysis (15-17) because α factor proteolysis occurs more rapidly at higher cell concentrations (16). To determine the exact extent of α factor inactivation, the disappearance of α factor activity was measured under the conditions of Fig. 2 using the agglutination assay. The disappearance of α factor activity was first order in all cases for at least three half-lives (Fig. 6). Such kinetics are expected for an enzymic reaction where the substrate concentration is at least 100 times smaller than the K_m value and where there are no cooperativity effects (43). The initial concentration of α factor used in these experiments (10^-8 M) is well below the reported K_m of 2.4 × 10^-5 M for α

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^1 Portions of this paper (including "Materials and Methods," part of "Results," Figs. 5-7, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, Bethesda, MD 20814. Request Document No. 83M-602, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
factor proteolysis by X2180-1A cells in YPD medium (16).

First order rate constants for α factor inactivation at various average cell concentrations were calculated as described under "Materials and Methods." The plot of k_{obs} against cell density was linear, and the slope yielded the second order rate constant for α factor inactivation, 

\[ k_i = 6.5 \times 10^{-9} \text{C}^{-1} \text{min}^{-1} \]

where C = cells/ml.

The fraction (F) of α factor remaining was calculated for the experiment of Fig. 2 using this second order rate constant and Equation 1,

\[ F = e^{-k_i t} \]

where t is the time of assay in minutes and C is the average cell concentration during the assay. At initial cell concentrations of 10^6 and 10^7 cells/ml, there is 0.9998 and 0.996 α factor remaining, respectively, after 3 h. That is, there is essentially no α factor destruction during the assay where the K_{50 (arrest)} is constant versus cell concentration. At 10^6, 10^7, and 10^8 cells/ml, there is 0.86, 0.21, and 2 × 10^{-7} α factor remaining, respectively, after 4 h. Thus, there is a significant loss of α factor activity at the time of assay where the K_{50 (arrest)} increases with increasing cell concentration.

These data are consistent with the K_{50 (arrest)} values above 10^6 cells/ml being dominated by the kinetics of α factor inactivation. The K_{50 (arrest)} value below 10^6 cells/ml must reflect some other process because α factor destruction is insignificant at these low cell concentrations. The K_{50 (arrest)} values measured at 10^6 cells/ml, where there is no α factor inactivation during the assay, are given in Table I for various cell strains and culture conditions.

K_{50} Values for α Factor-induced Agglutination of MATa Cells—Preincubation of MATa cells with α factor increased their agglutinability with nongrowing tester MATa cells in all cell strains tested (Table III). The dose-response curve for α factor induction of agglutinability is shown in Fig. 3. The K_{50} values obtained from dose-response curves for various cell strains and assay conditions are given in Table I. The independence of K_{50} for 381G cells on the change in cell density between 1 and 4 × 10^7 cells/ml and the lack of any effect by the addition of 10 mM TAME into the assay (Table I) indicate that no significant α factor inactivation is occurring in the assay for this particular cell strain. TAME is a protease inhibitor which inhibits α factor destruction (15).

The K_{50} values for other cell strains are significantly lowered by the addition of 10 mM TAME with α factor (Table I), indicating that α factor inactivation is occurring in these cases. TAME at 10 mM had no effect on the cell doubling time.

The addition of cAMP, trypsin- and pepsin-inactivated α factor, luteinizing hormone, and tunicamycin had no effect on the K_{50} (Table I, footnotes). Therefore, no effect of the agglutination response has yet been found, whereas several of the above compounds do effect the shmoo cell response (26, 44).

K_{50} for α Factor-induced Projection Formation—Cells which are arrested for division by α factor grow large and form unusual shapes consisting of one or more large pointed projections extending from the cell body. This is referred to as the shmoo morphology (20, 22, 23). It was found here that projections are formed at high (≥10^{-8} M) but not low (<10^{-9} M) α factor concentrations (see Fig. 7). At 4 h in 4 nM α factor, cell division is arrested and ≥95% of the cells are

The abbreviations used are: TAME, N\textsuperscript{6}-p-tosyl-L-arginine methyl ester; of, α factor; LH, luteinizing hormone; TLME, N\textsuperscript{6}-p-tosyl-L-lysine methyl ester; YNB, yeast nitrogen base.
ml where no α factor destruction occurs during the assay. A similar K_{50(arrest)} value was obtained when projection formation was assayed at 4 h after the addition of α factor, although the experimental error in this case was larger because projections were smaller and more difficult to monitor.

α factor concentrations of less than 5 nM gave no observable projections after 7 h (Fig. 4). This means either there is a threshold α factor concentration below which projection formation cannot occur, or the assay is not sensitive enough to detect small projections or associated morphological changes which may occur below 5 nM α factor. In order to obtain a linear double reciprocal plot for projection formation, it was necessary to correct the α factor concentration as described in the legend of Fig. 4.

DISCUSSION

The Mechanistic Determinant of K_{50(arrest)} at Different Cell Densities—The K_{50(arrest)} is dependent on cell concentration above 10^5 cells/ml, and independent of this parameter below 10^5 cells/ml (Fig. 2). This is because different biochemical effects determine the kinetics of cell division arrest and recovery, and therefore the observed K_{50(arrest)} value, at high and low cell densities.

At 10^5 cells/ml, the K_{50(arrest)} is very high at 2 × 10^{-2} M (Fig. 2). From the second order rate constant for α factor inactivation, k_i = 6.5 × 10^{-9} C^{-1} min^{-1}, it can be calculated that at the 4-h time of assay there is 2 × 10^{-5} of the original α factor concentration remaining (8 × 10^{-4} at 3 h). That is, nearly all of the α factor has been destroyed at the time of assay. This indicates that the quantitative value of the K_{50(arrest)} at 10^5 cells/ml is determined predominately by the mechanism of inactivation of α factor by the cell-associated protease previously reported (15–17). It is noteworthy that the value of k_i, which was determined here by the loss of α factor activity in the agglutination assay, is in good agreement with the values of k_i which I was able to calculate from published data which directly measure the proteolytic cleavage of α factor. These values are 2.3 × 10^{-9} C^{-1} min^{-1} for X2180-1A cells in SD medium, 25 °C (15) and 2.6 × 10^{-10} C^{-1} min^{-1} for X2180-1A cells in YPD medium, 23 °C (16).

Below 10^5 cells/ml, the K_{50(arrest)} of 2.5 × 10^{-10} M is independent of cell density (Fig. 2). This observation alone strongly suggests that α factor inactivation by the cell-associated protease is not a dominant parameter in the kinetics of arrest below 10^5 cells/ml because decreasing the cell (and therefore the cell-associated protease) concentration has no effect on the observed K_{50(arrest)} value. Consistent with this is the calculation from the k_i value that 9.98 or more of the original α factor concentration is present at the 3-h time of assay at cell densities below 10^5 cells/ml. In fact, at 1 nM α factor and 10^5 cells/ml when most or all of the cells had recovered and recommenced cell division, the old “conditioned” medium minus the original cells was capable of arresting fresh cells with identical kinetics of arrest and recovery as the original cell population (45). This demonstrates unequivocally that there was no significant inactivation of α factor activity at 10^5 cells/ml under these conditions. It is concluded that cells can recover from arrest without α factor inactivation by becoming insensitive, or desensitizing, to α factor (45). Thus, the kinetics of arrest and recovery at 10^5 cells/ml represents this slow, rate-determining recovery by desensitization (45).

The K_{50(arrest)} values in the intermediate range of 10^3–10^5 cells/ml are expected to be dependent on both recovery by α factor inactivation and recovery by desensitization, with α factor inactivation predominating at the higher and desensitization predominating at the lower cell concentrations.


Protease and Protease Inhibitors—Studies on cell division arrest in the absence of significant a factor destruction can probably be achieved at higher cell densities using the bar1 (39) or sst1 (40) mutant that is deficient in the protease which inactivates a factor. Alternatively, where it is desirable to use strains previously reported, or wild type strains, the modified forms of a factor in which Lys-7 is replaced by D-Lys or Arg may be used. These forms of a factor are inactivated very slowly or not at all (28, 41).

The protease inhibitor TAME was found (data not shown) to potentiate the division arrest response of a factor (i.e. lower the K_{a0,rest}), consistent with previous reports (5, 15). However, TAME at concentrations where growth was unaffected (≤10 mM) did not completely eliminate a factor destruction in the arrest assay which takes 3–4 h. The inability of TAME to eliminate a factor destruction at these concentrations is due in part to the fact that TAME is hydrolyzed by the proteases it inhibits (see Ref. 15).

On the other hand, TAME does appear to completely inhibit a factor destruction in the agglutination assay which takes only 1 h and has a half-time for induction of agglutination of only 20 min (31, 32, 34). The varying effects of 10 mM TAME in the agglutination assay (Table I) may be caused by different amounts of active protease in the various cell strains. By this criterion, 381G would have the lowest level of active protease because the K_{aggl} is not affected by TAME. Nevertheless, 381G does destroy a factor (Fig. 6).

Independence and Interdependence of Mechanisms—It is clear that arrest and agglutinin induction by a factor must involve mechanisms which are independent of both significant a factor inactivation and a-factor-induced and fully formed projections because these are eliminated in the assays where complete arrest and agglutination induction are observed. The data do not rule out the possibility that a very small amount of a factor inactivation and the occurrence of small scale biochemical changes associated with projection formation are required for complete arrest and agglutinin induction. Shmoo formation has been reported to be independent of a factor inactivation based on the observation that the inhibition of a factor inactivation by chloroquine produces a proportional increase in the production of shmoo cells (16). The observation that arrest and agglutinin induction by a factor occur with nearly identical K_{a0} values for 381G cells (Table I) is consistent with a common a factor saturable site, or receptor, inducing both arrest and agglutinin production in MATα cells. Recently binding studies have indicated the existence of a receptor for a factor on the surface of MATα cells (42), and there is genetic evidence for a receptor (35). On the other hand, the observation that the K_{a0,proj} is approximately 2 orders of magnitude higher than the K_{a0} value for arrest and agglutination induction in the absence of inactivation of the a factor suggests that a different system of lower affinity mediates projection formation. If the same system and receptor are used, a much higher occupancy is required for the induction of projection formation compared to the induction of division arrest and agglutination.

In contrast to the results described here, it has been reported that the concentration of a factor needed to induce agglutination in normal MATα cells is 10^(-10)^-fold lower than that needed to inhibit DNA synthesis (31) or arrest cell division (4). The results described here indicate that the previous results on cell division arrest required higher a factor concentrations either because of the occurrence of a factor inactivation at the high cell density used (31) or because the a factor concentration which induced shmoo formation was erroneously taken to be identical with that needed to induce cell division arrest (4).

The Temporal Sequence of Events Induced by a Factor—It has been claimed that the earliest detectable response of MATα cells to a factor is the increase in agglutinability towards MATα cells, after which cell division arrest occurs (4, 5, 31). Other workers (3) have made the opposite claim. It was found here at saturating a factor concentrations that whole populations of exponentially growing cells complete the induction of agglutinin in response to a factor (t_{1/2} = 20 min) (31, 32, 34) before all of the cells traverse the cell cycle and display cell division arrest (t_{a0} = 90 min) (Fig. 5B), as previously described (6). However, individual cells just prior to the a factor execution point arrest cell division and induce agglutination in response to a factor nearly simultaneously.

The half-time for induction of agglutination at saturating a factor concentrations is about 20 min (31, 32, 34). In comparison, individual cells near the a factor execution point (the cdc28 step (10)) arrest cell division at saturating a factor (i.e. lower K_{a0}) nearly simultaneously. The observation that arrest and agglutination induction by a factor must be independent of one another is consistent with previous reports (39) or sst1 (40) mutant that is deficient in the protease which inactivates a factor (Ref. 15). How-
a Factor-induced Responses in Yeast

Supplemental material to compare DOOR-RESISTANCE FOR AFA POPULATION INCREASE DIVISION ARREST, MANIFESTED AS PRODUCTION OF TRAPP CELLS: IMPLICATION FOR MECHANISM OF AFA FACTORS ACTION.

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Materials and Methods

Materials. Saccharomyces cerevisiae Applied Strain Used were 1762 BMH-3-11C (strains used in this study), 1761 BMH-3-9C, 1763 BMH-3-9C, and 1763 BMH-3-11C. All strains were grown in YNB media supplemented as described below. Cells were grown in the presence of 0.5% NaCl to a density of at least 10^8 cells/ml. The samples were collected by centrifugation at 3000 x g for 5 min at 4°C. The pellets were resuspended in the required buffer and were used immediately. Unless otherwise stated, all experiments were performed with cells from one culture. The cultures were grown in the presence of 0.5% NaCl.
Figure 1 shows the cell number and %LD responses after alpha factor addition. After the addition of alpha factor, the cell number continues to increase as cells progress through the cell cycle and divide. A plateau is reached where there is an invasor slow cell number increase over time. Cell number begins to rise again at a well-defined time as cells recover from arrest and resume division. At lower alpha factor concentrations, the kinetics are similar or identical except the time of initial recovery occurs earlier (16). After alpha factor addition, the cell number begins to rise again at a well-defined time as cells recover from arrest and resume division. At lower alpha factor concentrations, the kinetics are similar or identical except the time of initial recovery occurs earlier (16).

Figure 4 shows the decay in alpha factor activity over time, which is first order. This plot yields a rate constant for alpha factor inactivation, $k_{inact} = 1.6 \times 10^{-10}$ M$^{-1}$ min$^{-1}$ at 0.2 x 10$^9$ cells/mL in 1 N NaOH at 30°C. At the time indicated, 0.1 ml aliquots were removed, made to 0.2 M NaOH, incubated for 5 min, centrifuged for 10 min, adjusted to 400 x g, and the supernatant frozen at -70°C. These samples were later assayed in triplicate for alpha factor activity using the equilibration assay.

Figure 6 shows the cell morphology at two alpha factor concentrations and two durations. Cultures of 10$^9$ cells/mL in 200 ml of prefiltered YPD + 3% glucose, pH 5.5, 30°C were incubated with alpha factor at 100 mM at 400 x g for 3 hours. Aliquots were removed and added to 0.2 M NaOH at indicated times, centrifuged, resuspended and photographed.