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Role of Metabolism of the Mating Pheromone in Sexual Differentiation of the Heterobasidiomycete *Rhodosporidium toruloides*

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A trypsin-type endopeptidase (Kamiya et al., Biochem. Biophys. Res. Commun. **94**:855–860, 1980) responsible for the metabolism of rhodotorucine *A*, the farnesyl undecapeptide mating pheromone secreted by mating type *A* cells of *Rhodosporidium toruloides*, was biologically characterized. Metabolic activity was found to be present exclusively on the cell surface of the pheromone target cell. The activity was highly specific to the pheromone, and a biologically inactive analog which has the complete amino acid sequence of rhodotorucine *A* but lacks the farnesyl residue was not metabolized by intact cells. Pheromone metabolism was inhibited by trypsin substrates such as tosyl-L-arginine methyl ester. The presence of tosyl-L-arginine methyl ester strongly inhibited the sexual differentiation induced by the pheromone at a concentration which did not affect the vegetative growth of *R. toruloides*. Pheromone-induced sexual differentiation was also strongly inhibited by a metabolizable analog, rhodotorucine *A* S-oxide, but not by a non-metabolizable one. In mutants defective in early processes of mating, the decrease in the pheromone metabolic activity correlated well with the extent of loss of sensitivity to the pheromone. Both the pheromone metabolism and the capacity for sexual differentiation of a sterile mutant were restored concomitantly with reversion from the sterile to the fertile phenotype. These results suggested that metabolism of the mating pheromone plays an essential role in the process of sexual differentiation in *R. toruloides*.

Conjugation between two haploid cells of compatible mating types, *a* and *A*, of the heterobasidiomycetous yeast *Rhodosporidium toruloides* is mediated by a mating-type-specific diffusible mating pheromone secreted by the haploid cells of each mating type (1). The pheromone elicits sexual differentiation from the target cell, which serves as a prelude to mating, arresting the normal vegetative growth in the G1 phase of the cell division cycle (1; K. Abe, Ph.D. thesis, University of Tokyo, 1979). The morphological changes induced by the mating pheromone are conspicuous; an elongated "mating tube" (1) is observed, as compared with the "schmoo" formation in the yeast *Saccharomyces cerevisiae* (5). Biochemical characterization of the sexual differentiation of *R. toruloides* has not been attempted yet; however, on the basis of our data obtained with the study of an analogous system, *Tremella mesenterica* (another species of the heterobasidiomycetous yeasts; 7, 8, 22; E. Tsuchiya, Ph.D. thesis, University of Tokyo, 1979), sexual differentiation in *R. toruloides* possibly involves various biochemical alterations, such as inhibition of DNA synthesis and change in cAMP level, and the pheromone eventually modulates the gene expression of the target cell.

The chemical structure of rhodotorucine *A*, the mating pheromone produced by the mating type *A* cells of a strain of *R. toruloides*, has been elucidated to be a lipopeptide, namely, farnesyl undecapeptide (Fig. 1A) (10). As low as nanomolar concentrations of the pheromone induce sexual differentiation in target (mating type *a*) cells (10). In contrast to the simple peptide structure of the α- (12, 18) and **a**- (3) mating factors of *S. cerevisiae*, all mating pheromones of the heterobasidiomycetous yeasts so far characterized have lipopeptide as a common structural feature (rhodotorucine *A* [10], tremerogen *A*-10 [16], and tremerogen *a*-13 [17]). Experiments with chemically synthesized rhodotorucine *A* and its analogs have suggested that rhodotorucine *A* is a signal substance sufficient for eliciting sexual differentiation from the target cell, and

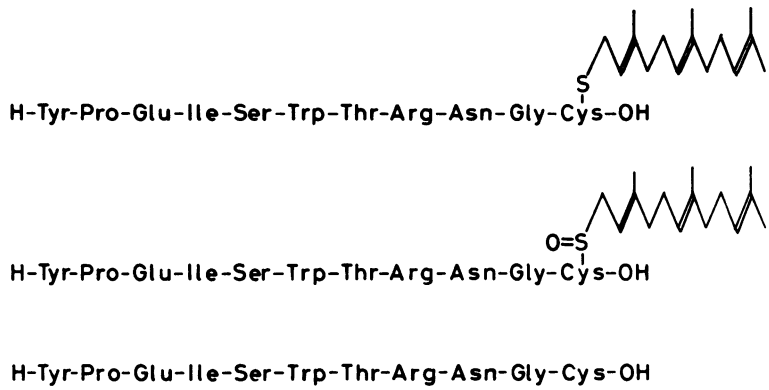

FIG. 1. Chemical structure of rhodotorocine *A* and analogs used: (A) rhodotorucine *A*, (B) rhodotorucine *A* S-oxide, and (C) SH-rhodotorucine *A*.

that the structure involving both peptidyl and farnesyl moieties is indispensable for its function (21).

The elucidation of the chemical and topological fate of the pheromone at the cell surface of the target cell is a vital problem for the understanding of the mechanism of transmembrane signaling for sexual differentiation. For this purpose, we initiated studies on the interaction of rhodotorucine *A* with the target cell. During the course of our studies on the binding of labeled rhodotorucine *A* to the target cell, we noted that the amount of pheromone bound to the cell was only a small proportion of the amount administered, though the biological activity of the pheromone was eliminated exhaustively from the incubation medium. This discrepancy was explained by the observation by Kamiya et al. (9) and by us (13) that rhodotorucine *A* is metabolized by a specific hydrolytic activity of the target cell. This metabolism, however, has not yet been biochemically characterized. In this communication, we describe the properties of the metabolism of the mating pheromone and present data which strongly suggest that this metabolism plays an essential role during sexual differentiation in *R. toruloides*.

MATERIALS AND METHODS

Microorganisms and growth conditions. Haploid strains of *R. toruloides* IFO 0559-M-919 (mating type *A*, Met$^-$ Pan$^-$) and IFO 0880-M-1057 (mating type *a*, PABA$^-$), described in our previous paper (1), were used for most of the studies described. Haploid strains of *Tremella mesenterica*, IFO 9310 (mating type *AB*) and IFO 9313 (mating type *ab*), (21), were also used. Cells were routinely grown by reciprocal shaking at 28°C in a 500-ml flask with 100 ml of YPG medium containing 0.4% yeast extract, 0.5% polypeptone, 2% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 50 μg of chloramphenicol per ml. For preparation of rhodotorucine *A*, GP medium, containing 2% glycerol, 0.7% polypeptone, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 50 μg of chloramphenicol per ml and adjusted to pH 6.5 by the addition of 1 M sodium phosphate buffer (pH 7.0), was used.

Isolation of sterile mutants and fertile revertants. Mutants defective in an early process of mating, i.e., cells which do not form a mating tube in response to rhodotorucine *A*, were isolated by the following procedure. Cells were mutagenized with 3% ethyl methane sulfonate for 90 min by the method of Rasse-Messenguy and Fink (14). Survival after ethyl methane sulfonate treatment was about 30%. The treated cells were suspended in YPG, grown for 24 h, and plated on YPG agar plates containing 300 U of the mating pheromone to yield ca. 300 colonies per plate. After 48 h of incubation at 28°C, small colonies appeared on the plate. Wild-type colonies were fluffy, due to mating tubes forming on the pheromone-containing plates; mutant cells were picked with a toothpick from the smooth colonies. These mutant cells were purified by a single-colony isolation technique, and the genetic marker derived from the parental strain was confirmed. Twenty pheromone-insensitive strains were obtained from 60,000 colonies tested.

Fertile variant strains, in which the ability to differentiate in response to the pheromone was restored, were screened and isolated from a sterile mutant (strain 39). The principle of the screening method is based on the tendency of the mating tube to penetrate into the agar layer of plated culture medium containing a high concentration of the pheromone; the tubed cells are thus difficult to remove with a rubber policeman under conditions in which most of the vegetative cells are removed. Fertile variants were isolated by the following procedure. Cells were plated on a YPG agar plate, containing 150 U of the mating pheromone per ml, at a density of 1.5×10^7 cells per plate and allowed to grow overnight at 28°C. After undifferentiated cells were swept away from the surface of agar medium by the rubber policeman method described above, cells having a mating tube were picked from the agar plate by using a toothpick under a phase-contrast microscope. These cells were then purified on a pheromone-containing plate by the single-colony isolation method. Nine fertile strains were isolated from five petri dishes. The actual frequency of reversion was unknown since the yield rate of revertant strains was low with the procedure used.

Preparation of rhodotorucine *A* and analogs. Practi-

cally pure rhodotorucine *A*, prepared by the procedure described by Kamiya et al. (10), was used for the preparation of ^{125}I-labeled rhodotorucine *A* and rhodotorucine *A* S-oxide. The mating pheromone, when judged pure by thin-layer chromatography (TLC) and high-performance liquid chromatography, usually had a specific biological activity of 0.125 U per ng of the preparation. Partially purified rhodotorucine *A* was used in most of the experiments in which pheromone metabolism was determined by the elimination of the biological activity of the mating pheromone. The purification procedure comprised the following steps: (i) precipitation of rhodotorucine *A* by addition of ammonium sulfate (80%) to the culture filtrate of mating type *A* cells grown to the late logarithmic phase of growth; (ii) extraction of the pheromone with *n*-butanol from aqueous solution; and (iii) gel filtration through a column of Sephadex LH-20 (3.5 by 11 cm) with methanol as the developing solvent, followed by concentration of the active fraction in a rotary evaporator. Isotopically pure ^{35}S-labeled rhodotorucine *A* and rhodotorucine *A* S-oxide were prepared by the procedure described by Kamiya et al. (9). Chemically synthesized SH-rhodotorucine *A* was a generous gift from M. Fujino.

Assay of the biological activity of rhodotorucine *A*. The estimation of rhodotorucine *A* was conducted by the following procedure. A piece of agar film (3 by 3 mm, 0.7 mm thick) containing approximately 1,000 *a* cells was soaked in 0.5 ml of twofold serial dilutions of the pheromone, and the culture was allowed to incubate at 28°C for 6 h with gentle shaking. The agar piece was removed, and the percentage of cells with a mating tube was determined under a microscope. One unit of activity per ml was defined as the pheromone concentration which gave the characteristic morphological change in 30% of the assay cells under the above conditions.

Radioiodination of rhodotorucine *A*. Rhodotorucine *A* was routinely iodinated with carrier-free Na^{125}I at 0°C by lactoperoxidase-catalyzed reaction in the presence of peroxide generated by glucose and glucose oxidase. A mixture containing 1.4 μg of pure pheromone, 10 μCi of carrier-free Na^{125}I, 0.03 U of glucose oxidase, and 0.1 μg of lactoperoxidase in 50 μl of 50 mM sodium phosphate buffer (pH 7.0) was placed in an ice bath. The reaction was initiated by adding 5 μl of 5 mM glucose and mixing well. The same amount of glucose was added four more times at intervals of 1 h. At the end of 1 h of incubation after the last addition of glucose, 10 μl of 1 M 2-mercaptoethanol was added to terminate the reaction. One milliliter of water was added to the reaction mixture, and the labeled pheromone was extracted with 1 ml of *n*-butanol. After the *n*-butanol layer was washed several times with water, an isotopically pure preparation of radioiodinated pheromone was obtained from the *n*-butanol layer. After evaporation of the *n*-butanol in vacuo, the labeled pheromone was dissolved in 100 μl of methanol and stored at −20°C. The labeled preparation usually had a specific radioactivity of 1,500 cpm/ng. Analogs of rhodotorucine *A* were iodinated and purified by a method similar to the above except that ^{125}I-labeled SH-rhodotorucine *A* was purified by preparative TLC on a Silica Gel G plate in solvent A (*n*-butanol–acetic acid–water, 4:1:5 by volume, upper layer). In some instances, to increase the percentage of iodinated species, iodination was carried out in the presence of an excess amount of carrier NaI (0.3 μg) added to the reaction mixture described above.

Analysis of metabolic products of rhodotorucine *A*. ^{125}I-labeled rhodotorucine *A* or analogs (10 ng, 10,000 cpm) were incubated for 10 min at 28°C in a suspension of mating type *a* cells made up in 0.5 ml of 1 mM sodium phosphate buffer (pH 7.0) at a concentration of 1.2×10^8 cells per ml. After removal of the cells by pelleting in a Microfuge, the supernatant fraction was concentrated in vacuo. The dried material was dissolved in 50 μl of methanol and subjected to two-dimensional (2-D) TLC on a Silica Gel G thin-layer plate (Kiesel Gel 60, Merck) developed by ascending chromatography in solvent A (*n*-butanol–acetic acid–water, 4:1:5 by volume, upper layer) for the first dimension and solvent B (phenol–water, 3:2 by volume) for the second dimension. The gel was dried and exposed to Kodak X-Omat R film with an intensifying screen (Lightning-Plus, Dupont) at −80°C for 1 to 2 days. Metabolic products of ^{35}S-labeled rhodotorucine *A* were analyzed by the procedure described above with 5,000 cpm of purified [^{35}S]rhodotorucine *A*. This amount of labeled pheromone had 1 U of biological activity, which is equivalent to 8 ng of pure pheromone. Radioactive spots were detected by exposing the dried gel to Kodak X-Omat R film for 3 weeks. Tryptic digestion of rhodotorucine *A* and analogs was performed by incubating the labeled compound with 50 μg of trypsin and 5 μg of tosylamide-2-phenylethyl chloromethyl ketone in 500 μl of 1 mM sodium phosphate buffer (pH 6.0) for 2 h at 28°C; the compound was then analyzed by the above method.

Chemicals and isotopes. Chemicals were obtained from the following sources: phenylmethylsulfonyl fluoride, chloroquine, aprotinin, soybean trypsin inhibitor, trypsin, lactoperoxidase, and bovine serum albumin were from Sigma; tosylamide-2-phenylethyl chloromethyl ketone and *p*-tosyl-L-arginine methyl ester (TAME) were from Tokyo Kasei; benzoyl-L-arginine β-naphthylamide was obtained from Nakarai; glucose oxidase came from Boehringer Mannheim; and yeast extract and polypeptone were obtained from Daigo Eiyo. All radioactive chemicals used were purchased from Amersham.

RESULTS

Elimination of rhodotorucine *A* activity by the target cell. As reported in our previous paper (20), when a solution of rhodotorucine *A* was incubated in a suspension of mating type *a* cells, pheromone activity diminished very rapidly during the incubation period (Fig. 2). In this particular experiment, 200 U of the original pheromone activity decreased to 14 U within 5 min of incubation at 28°C. The rate of decrease varied from experiment to experiment, depending especially on the purity of the mating pheromone used and the growth phase of the cell. The pheromone activity was eliminated by the cell at a substantial rate even in a 0°C incubation (Fig. 2).

Binding of labeled rhodotorucine *A* to the target cell. To explore the fate of the pheromone during

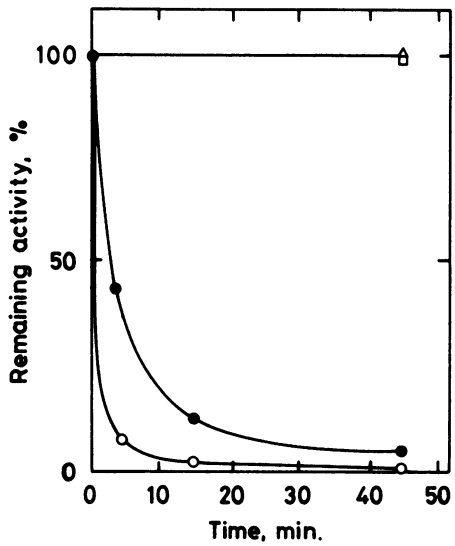

FIG. 2. Elimination of rhodotorucine *A* activity by incubation with mating type *a* cells. Two hundred units of partially purified rhodotorucine *A* was incubated at 28°C with 3×10^7 cells of mating type *a* (○) or mating type *A* (△) in 1 ml of YPG with gentle shaking. Incubation with mating type *a* cells was also carried out at 0°C (●). At the time indicated, cells were removed by pelleting for 1 min in a Microfuge, followed by 1-min boiling of the supernatant fraction to kill the remaining cells. The biological activity of mating pheromone remaining in the boiled preparation was determined by the serial dilution method described in the text. Partially purified pheromone was also incubated with 1 ml of the culture filtrate of *a* cells. The reaction was terminated by 1 min of boiling, and the residual pheromone activity was determined as described above (□).

interaction with the target cell, we prepared ^{125}I-labeled rhodotorucine *A*. The biological activity of the pheromone was found to be very sensitive to oxidative conditions of iodination. Using conditions described in Materials and Methods, however, we succeeded in preparing labeled pheromone with a specific biological activity comparable to that of native pheromone. An autoradiogram of a TLC plate of a typical preparation of [^{125}I]rhodotorucine *A* is shown in Fig. 3. Labeled species were not resolvable from native pheromone by the TLC system used.

The time course of binding of the labeled pheromone to mating type *a* cells is shown in Fig. 4. Cell-bound radioactivity increased very rapidly and reached maximal binding within 6 min of incubation at both 0 and 28°C; the bound radioactivity subsequently decreased, reaching a plateau level in 30 min. The rate of the decrease in the bound radioactivity was much faster at 0°C. The amount bound to the cell was only about 6% of the radioactivity administered to the suspension even at maximal binding (6 min). The amount associated with the cell at the plateau level (30 min) was about 67% of the maximal binding (6 min) with incubation at 28°C, whereas only 15% of the initially bound radioactivity remained associated with the cell at 0°C (Fig. 4). A similar low level of binding (6% of the radioactivity administered) was also demonstrated with [^{35}S]rhodotorucine *A* labeled at the carboxy-terminal cysteine residue, the opposite end from the ^{125}I label of the iodinated pheromone. To explore the cause of the unusual kinetics of the binding reaction, the properties of the radioactive substance which remained in the incubation medium were examined. When fresh *a* cells were added to the supernatant fraction isolated after incubation of the labeled pheromone with *a* cells, practically no binding of the radioactivity to the cells was seen (Table 1). Accumulation of binding inhibitors in the medium during the previous incubation was not the cause, since labeled pheromone, when added to the supernatant fraction together with fresh *a* cells, bound to the cells to the same extent as in the first incubation (Table 1). From these results, a conclusion was drawn that the elimination of the biological activity of the pheromone during incubation with the target cells was mainly due to biochemical modification of the pheromone by the cell to a form that lacked binding activity.

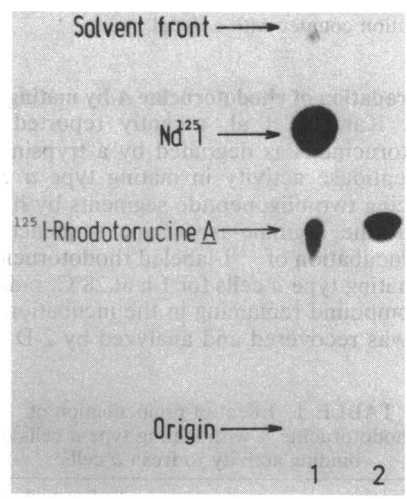

FIG. 3. Thin-layer chromatogram of [^{125}I]rhodotorucine *A*. The separation was on a Silica Gel G thin-layer plate in solvent A, and the radioactive components were visualized by autoradiography. Lane 1, Sample spotted directly from the reaction mixture after termination of iodination reaction; lane 2, sample spotted after purification by extraction to the *n*-butanol layer from the water layer, followed by extensive washing of the *n*-butanol layer with water. The labeled species was not resolvable from native pheromone by the TLC system.

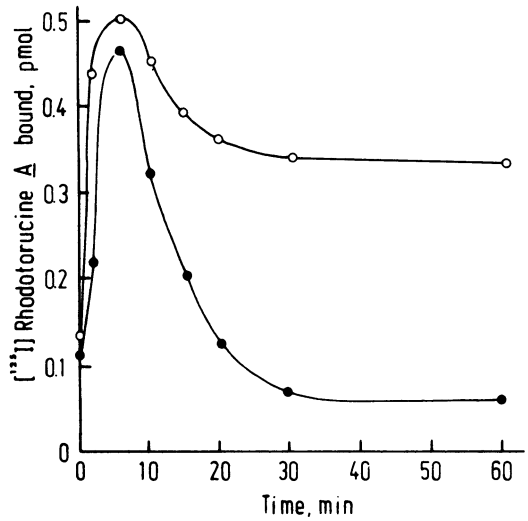

FIG. 4. Time course of binding of [^{125}I]rhodotorucine *A* to mating type *a* cells. Mating type *a* cells were suspended in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 0.05% of bovine serum albumin at a cell concentration of 2×10^7 cells per ml. The binding reaction was initiated by the addition of ^{125}I-labeled pheromone (15 ng, 15,000 cpm). The incubation was carried out at either 28°C (○) or 0°C (●). At the indicated time, cells were collected quickly on a fiber glass filter (CG 50, Toyo Kagaku) and washed with 10 ml of the above buffer containing bovine serum albumin at 0°C. The collecting and washing procedure was completed within 1 min. The radioactivity remaining in the filter disk was determined in a liquid scintillation counter with a γ-vial.

Degradation of rhodotorucine *A* by mating type *a* cell. Kamiya et al. recently reported that rhodotorucine *A* is degraded by a trypsin-type endopeptidase activity in mating type *a* cells, producing two oligopeptide segments by hydrolysis at the arginine-asparagine sequence (9). After incubation of ^{125}I-labeled rhodotorucine *A* with mating type *a* cells for 1 h at 28°C, radioactive compound remaining in the incubation medium was recovered and analyzed by 2-D TLC (Fig. 5). In agreement with the observation by Kamiya et al., after incubation of rhodotorucine *A* with *a* cells a new spot appeared at the same position as the trypsin cleavage product of [^{125}I]rhodotorucine *A*. A spot of iodide generated during the incubation can be seen in the top right part of the TLC pattern (Fig. 5). This spot appeared even in the control experiment, and we assumed it was not an important reaction for pheromone metabolism. The time course of the hydrolysis of the mating pheromone was consistent with that of elimination of the biological activity (data not shown), suggesting that hydrolysis is the direct cause of pheromone inactivation.

TABLE 1. Effect of preincubation of [^{125}I]rhodotorucine *A* with mating type *a* cells on its binding activity to fresh *a* cells

Expt	Suspension medium	Radioactivity (cpm)	
		Cell-bound	Free
1	[^{125}I]rhodotorucine *A*, 15,000 cpm	976	12,640
2	Supernatant from expt 1	62	11,550
3	Supernatant from expt 2	0	11,320
4	Supernatant from expt 3, supplemented with [^{125}I]rhodotorucine *A*, 15,000 cpm	985	24,270

Location of the site of rhodotorucine *A* metabolism. The cellular and subcellular distribution of the metabolic activity of the mating pheromone is summarized in Table 2. It shows that the activity is specific to mating type *a* cells of *R. toruloides* and is not secreted in the medium. These results are consistent with the observations by Kamiya et al. (9). Table 2 also shows the result of a preliminary investigation on the subcellular distribution of the activity. Mating type *a* cells were disrupted by a French press, and the metabolic activity in the supernatant and the pellet fractions obtained after centrifugation (30,000 × *g* for 20 min) was determined. When the metabolic activity was measured by the elimination of the biological activity of the pheromone, almost all the activity associated with the intact cells was recovered in the pellet fraction. Products formed by each fraction were analyzed by 2-D TLC with [^{125}I]rhodotorucine *A*. The patterns shown in Fig. 6 demonstrated that incubation of labeled pheromone with the

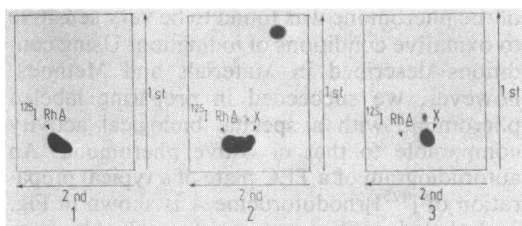

FIG. 5. In vivo metabolism of rhodotorucine *A* by mating type *a* cells. [^{125}I]rhodotorucine *A* (10 ng, 10,000 cpm) was incubated for 10 min at 28°C in the presence of (1) no cells and (2) 6×10^7 mating type *a* cells in 0.5 ml of 1 mM sodium phosphate buffer (pH 7.0). After removal of the cells by pelleting in a Microfuge, the supernatant fraction was boiled for 1 min in a water bath, lyophilized, and subjected to 2-D TLC as described in the text. The pattern of the trypsin cleavage product of [^{125}I]rhodotorucine *A* prepared as described in the text is also shown for comparison (3). The position of trypsin cleavage products is indicated by X.

TABLE 2. Cellular and subcellular distribution of rhodotorucine *A*-metabolizing activity

Incubation of pheromone with [a]:	Pheromone activity remaining (%)
Control[b]	100
R. toruloides	
Mating type *a* cells, 0°C	2.4
Mating type *a* cells, 28°C	0.8
Pronase-treated cells	1.6
Disrupted cells	0.5
Soluble fraction	50
Pellet fraction	0.5
Cell-free culture filtrate	100
Mating type *A* cells	100
T. mesenterica	
Mating type *ab* or *AB* cells	100

[a] Cells (3×10^7), or subcellular fraction prepared from the equivalent number of cells, were incubated with 200 U of rhodotorucine *A* in a 1-ml mixture for 1 h at 28°C unless otherwise indicated.

[b] Without cells or subcellular fraction.

pellet fraction yielded a product identical to that obtained by the intact cell. However, different products were obtained when incubated with the supernatant fraction. Thus, it was assumed that the metabolism is catalyzed by insoluble enzyme(s). A possible cell surface localization of the activity was suggested by the observation that the elimination of the pheromone activity proceeded very rapidly at 0°C incubation (Fig. 2 and Table 2). Under low temperatures, translocation of the pheromone molecule across the membrane should decrease dramatically; thus it is reasonable to assume that the rate of metabolism would be affected by incubation at low temperatures. For this reason, it is unlikely that rhodotorucine *A* was transported through the membrane and metabolized in the cytoplasm and that the metabolites were excreted into the medium. Our results suggested that the site of metabolism is outside the plasma membrane of the cell surface. However, treatment of the intact cells with a proteolytic enzyme, pronase, affected metabolic activity only slightly (Table 2). This result indicated that exposed cell surface proteins are not responsible for metabolism, but that the pheromone molecules must reach a pronase-inaccessible site of the cell surface to be metabolized. Further studies on the subcellular distribution of the pheromone-metabolizing enzyme have not been attempted yet because a method for preparation of *R. toruloides* protoplasts is not available.

Correlation of pheromone metabolism with the physiological response of cells to pheromone. To estimate the physiological role of pheromone metabolism during sexual differentiation, we examined the influence of inhibition of metabolic activity on the pheromone-induced sexual differentiation of *a* cells. Since trypsin-type endopeptidase was revealed to be responsible for metabolism of the pheromone, inhibitors of this metabolism were screened with an assay system described in the previous section, using labeled pheromone and, as substrate, inhibitors or substrates of various peptidases, especially trypsin-specific substrates. Among the reagents tested, TAME and benzoyl-L-arginine β-naphthylamide, both trypsin-specific substrates, were found to be effective inhibitors of pheromone metabolism (data not shown). Soybean trypsin inhibitor, phenylmethylsulfonyl fluoride, chloroquine, aprotinin, tosylamide-2-phenylethyl chloromethyl ketone, and several other chemicals tested (non-trypsin-type protease inhibitors) were all ineffective on the metabolic activity.

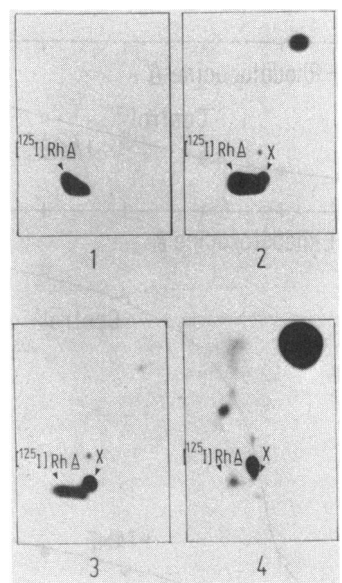

FIG. 6. Metabolism of rhodotorucine *A* by disrupted cells. Mating type *a* cells suspended in 4 ml of 1 mM sodium phosphate buffer (pH 7.0) at a cell concentration of 2×10^8 cells per ml were disrupted by a French press (Aminco) at 20,000 lb/in^2. After removal of undisrupted cells by low-speed centrifugation ($2,000 \times g$ for 5 min), the supernatant fraction was centrifuged twice at $30,000 \times g$ for 20 min (high-speed centrifugation) to obtain a clear cytoplasmic fraction. The pellet fraction after the first high-speed centrifugation was resuspended in the above buffer and centrifuged again as above. The resulting pellet was suspended in 4 ml of the above buffer. [^{125}I]rhodotorucine *A* was incubated in the presence of (1) no addition, (2) intact cells, (3) the pellet, or (4) the cytoplasmic fraction of the disrupted cells. The amounts of cells or subcellular fraction added in (2), (3), and (4) were all equivalent to 6×10^7 cells. The products were analyzed as described in the legend to Fig. 5. The direction of 2-D TLC was as denoted in Fig. 5. X indicates the position of the trypsin cleavage product.

The effect of metabolism inhibition by TAME on pheromone-induced sexual differentiation was investigated. Mating tube formation induced by the pheromone was strongly inhibited by the presence of 1 mg of TAME per ml (Fig. 7). Based on these data and the concentration-activity relationship of rhodotorucine *A*, it was estimated that the apparent biological activity expressed by 1 U of the pheromone per ml in the presence of TAME decreased to 0.02 U of the pheromone per ml. TAME at 1 mg/ml had no significant influence on the vegetative growth of *R. toruloides* (Fig. 7). Benzoyl-L-arginine β-naphthylamide, another metabolism inhibitor, strongly inhibited the vegetative growth of the cell and was excluded from the present study. The non-trypsin-type protease inhibitors tested had no effect on pheromone-induced sexual differentiation.

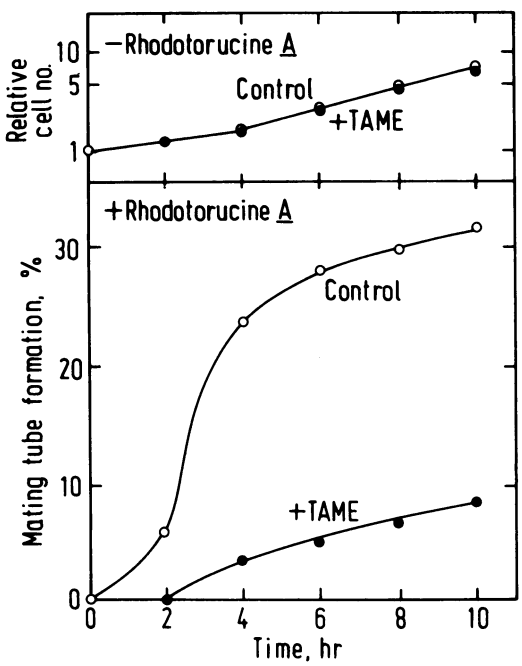

FIG. 7. Effect of TAME on the biological activity of rhodotorucine *A*. Approximately 1,000 cells (mating type *a*) solidified in a piece of agar film (3 by 3 mm, 0.7 mm thick) were incubated in 0.5 ml of YPG medium containing 1 U of rhodotorucine *A* per ml in the presence (●) or absence (○) of 1 mg of TAME per ml. After 1 h of incubation at 28°C with gentle shaking, the agar piece was taken out of the test tube, and the number of cells with mating tubes was counted quickly under a microscope. The agar piece was incubated again in a fresh medium containing the same additives as before. The procedure was repeated five times at intervals of 1 h (bottom). Similar experiments in the absence of rhodotorucine *A* was also carried out with (●) or without (○) 1 mg of TAME per ml to investigate the effect of TAME on the vegetative growth of the cells (top).

SH-rhodotorucine *A* (Fig. 1C), the peptide moiety of rhodotorucine *A*, has been shown to have no pheromonal activity and not to compete against the biological activity of native pheromone (21). When ^{125}I-labeled SH-rhodotorucine *A* was incubated with *a* cells, this analog was not significantly metabolized by the cells (Fig. 8). Since the ^{125}I label is at the amino terminus of the peptide, if it is metabolized by the trypsin-like activity a labeled metabolite identical to the ^{125}I-labeled rhodotorucine *A* metabolite should be produced from this analog (Fig. 7). These data suggest that the farnesyl residue is indispensible for the mating pheromone to be metabolized by the cell. Metabolism of another analog, rhodotorucine *A* S-oxide (Fig. 1B), an oxidized derivative of rhodotorucine *A* that has been reported to have no pheromonal activity (9), was also tested. When the ^{125}I-labeled analog was incubated with *a* cells, it was metabolized, producing a cleavage product identical to that of labeled rhodotorucine *A* (Fig. 8). The ^{125}I label of this analog is on a segment having a structure identical to that of the ^{125}I-labeled pheromone with respect to the trypsin cleavage site; evidently this analog was hydrolyzed by the same manner as native pheromone.

Both a metabolizable and a non-metabolizable analog of rhodotorucine *A* were found, and neither of them exhibited pheromonal activity. Thus, they were expected to serve as useful probes for the estimation of the physiological role of pheromone metabolism in sexual differentiation. The metabolizable analog, rhodotorucine *A* S-oxide, inhibited the activity of the native pheromone in a manner predicted from 1:1 competition (Fig. 9). On the other hand, the non-metabolizable analog, SH-rhodotorucine *A*, did not interfere with sexual differentiation at all, even at a concentration of 100-fold molar ratio excess over the native pheromone (Fig. 9). These data confirmed the result obtained with a trypsin inhibitor, that pheromone metabolism plays an important role in sexual differentiation.

Mating pheromone metabolism in sterile mutants. To explore the correlation of pheromone metabolism and sexual differentiation, the pheromone metabolism in sterile mutants derived from mating type *a* cells which failed to mate with *A* cells was examined. The sterile mutants used were described in a previous paper (2). Of 97 sterile strains isolated, strain 39 was the only one that was totally defective in the ability to eliminate the biological activity of the mating pheromone (Table 3). This defect in the mating pheromone metabolism by this strain was confirmed by failure in the proteolysis of ^{125}I-labeled pheromone (data not shown). This sterile mutant did not form a mating tube in the presence of the pheromone even at a concentra-

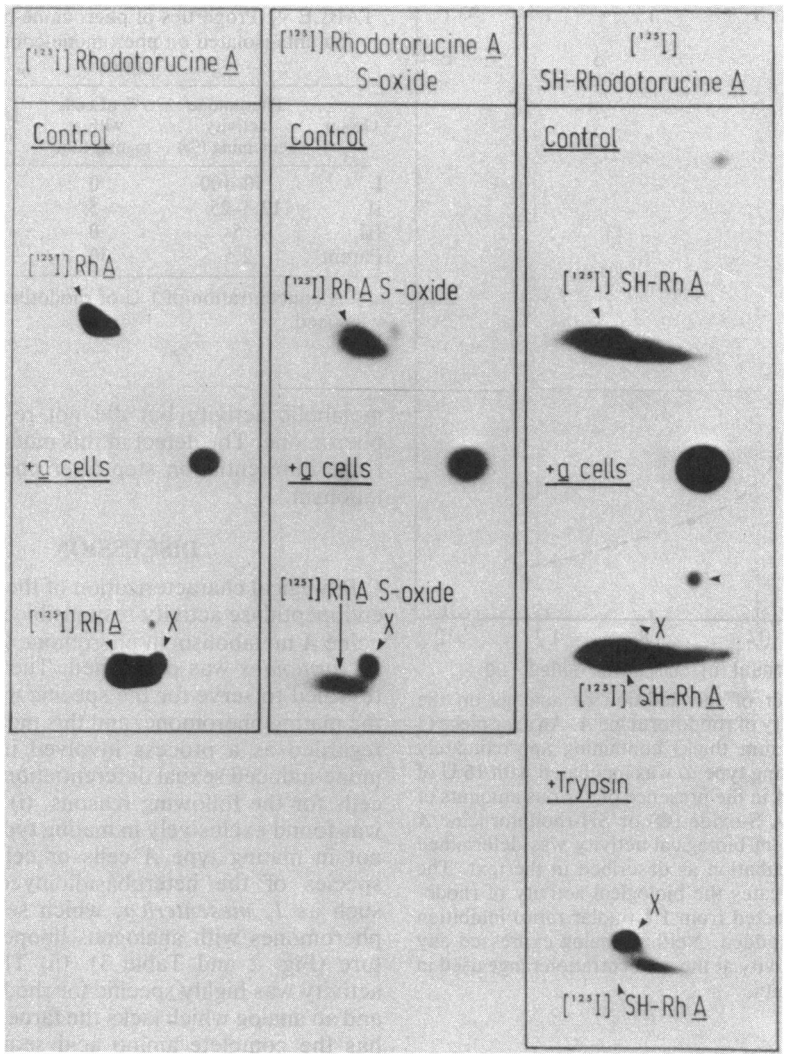

FIG. 8. In vivo metabolism of rhodotorucine *A* analogs by mating type *a* cells. ^{125}I-labeled rhodotorucine *A* analogs (15 ng, 15,000 cpm) were incubated with *a* cells and analyzed by 2-D TLC as described in the legend to Fig. 5. The direction of the chromatography was as denoted in Fig. 5. X indicates the position of the trypsin cleavage product of [^{125}I]rhodotorucine *A*. The pattern of trypsin-digested ^{125}I-labeled SH-rhodotorucine *A* is shown for confirmation of its chemical structure.

tion of 125 U of rhodotorucine *A* per ml. Fertile variants were isolated from sterile mutant 39 by the procedure described in Materials and Methods. All nine sterile strains examined regained all of the activities tested, such as mating ability, sensitivity to pheromone of mating tube formation, and the activity of pheromone metabolism, to the level of the parental strain (Table 3).

To further clarify the correlation of the genetic defect with pheromone metabolism and the biological response to the pheromone, mutants with a decreased response to the mating pheromone (i.e., which do not form mating tubes on agar medium containing a high concentration of rhodotorucine *A*), were isolated as smooth colonies, in contrast to the fluffy colonies characteristic of the wild type. Such mutants were expected to be defective in an early process of sexual differentiation. Mutants isolated by this method included strains with a "leaky" phenotype, which weakly responded to the pheromone. The properties of the mutants isolated are summarized in Table 4. All of the strains in group I, which have very weak pheromone metabolic activity (less than 4% of the parent), did not respond at all to 1 U of the pheromone per ml. Group II strains, which display weak but significant phermone metabolism, responded weakly to the pheromone, Al-

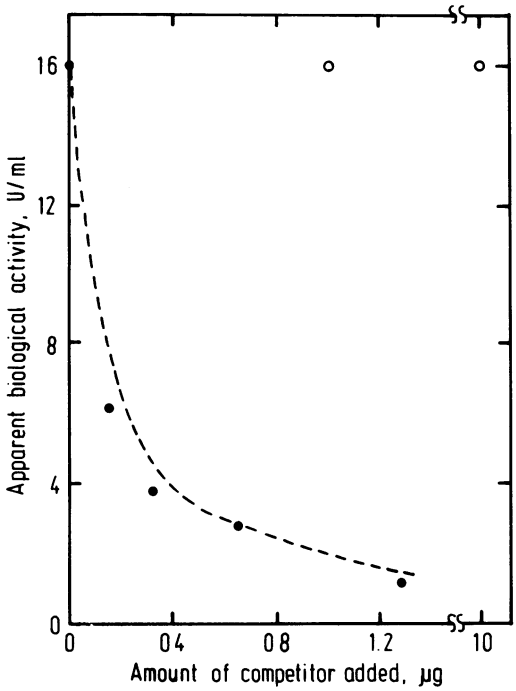

FIG. 9. Effect of the addition of analogs on the biological activity of rhodotorucine *A*. An agar piece (3 by 3 mm, 0.7 mm thick) containing approximately 1,000 cells (mating type *a*) was incubated with 16 U of rhodotorucine *A* in the presence of various amounts of rhodotorucine *A* S-oxide (●) or SH-rhodotorucine *A* (○). The apparent biological activity was determined after 6 h of incubation as described in the text. The dotted line indicates the biological activity of rhodotorucine *A* expected from 1:1 (molar ratio) inhibition by the analogs added. Neither analog expressed any pheromonal activity at the concentration range used in these experiments.

though the defective points of the mutants are unknown, a good correlation between in vivo metabolism and the extent of the pheromone response was seen. Among 16 strains tested, exceptions were the two strains of group III which had parental levels of the pheromone

TABLE 3. Response of a sterile mutant and its fertile revertant to rhodotorucine *A*

Strain	Mating ability	Concn of pheromone for 30% mating tube formation (U/ml)	Pheromone activity remaining (%)
Parent	+	1	7
Sterile mutant (strain 39)	−	≫125	100
Fertile revertant (strain 39-1)[a]	+	1	7

[a] The other eight revertant strains isolated were all similar to 39-1 in the three activities tested.

TABLE 4. Properties of pheromone-nonresponsive mutants isolated on pheromone-containing agar plates

Group	Pheromone activity remaining (%)	% of cells with a mating tube[a]	Mating ability	No. of strains isolated
I	50–100	0	−	5
II	12.5–25	5	±	9
III	5	0	−	2
Parent	2.5	30	+	

[a] A concentration of 1 U of rhodotorucine *A* per ml was used.

metabolic activity but did not respond to the pheromone. The defect in this mutant may be in some differentiation step after pheromone metabolism.

DISCUSSION

Biological characterization of the trypsin-type endopeptidase activity responsible for rhodotorucine *A* metabolism in pheromone target cells of *R. toruloides* was performed. The activity was revealed to serve for the specific metabolism of the mating pheromone, and this metabolism was regarded as a process involved in the pheromone-induced sexual differentiation of the target cells for the following reasons. (i) The activity was found exclusively in mating type *a* cells, but not in mating type *A* cells or cells of related species of the heterobasidiomycetous yeasts such as *T. mesenterica*, which secrete mating pheromones with analogous lipopeptidyl structure (Fig. 2 and Table 3). (ii) The metabolic activity was highly specific for rhodotorucine *A*, and an analog which lacks the farnesyl group but has the complete amino acid sequence of the native pheromone did not serve as a substrate for the metabolism in vivo (Fig. 8). (iii) Specific inhibition of the metabolism by a trypsin substrate (TAME) or a pheromone analog (rhodotorucine *A* S-oxide) also inhibited sexual differentiation, a specific response of the target cell to the pheromone (Fig. 7 and 9). (iv) Pleiotropic mutants of sterile phenotype defective both in an early process of sexual differentiation and in pheromone metabolism have been isolated. Metabolic activity was recovered concomitantly with reversion of the mutation to the fertile phenotype (Table 3). (v) In mutants defective in the early process of mating, a decrease in the pheromone metabolic activity correlated well with the extent of loss of sensitivity to the pheromone.

The site of the metabolism was located on the cell envelope. The enzyme was not accessible to pronase when intact cells were treated with this proteolytic enzyme (Table 2), however, suggest-

ing that it is not exposed to the medium. Our preliminary results indicated that a membrane enzyme is responsible for the metabolism, because the metabolic activity was effectively solubilized with a nonionic detergent, Nonidet P-40, in the pellet fraction of disrupted cells but not in intact cells, which are resistant to this detergent (K. Imai, unpublished data). Although the role of farnesyl residue in the process of signal transduction for differentiation is unknown, it is intriguing that, of the two biologically inactive pheromone analogs tested, rhodotorucine *A* S-oxide was metabolizable by the cell by the same manner as the native pheromone, but SH-rhodotorucine *A* was not (Fig. 8). This observation indicated that the farnesyl group is necessary for the substrate to reach the site of the enzyme in the hydrophobic environment of the membrane, or else the nonpolar group may play an essential role in recognition by the proteolytic enzyme. Further studies with the solubilized enzyme are in progress.

Rhodotorucine *A* S-oxide was metabolized by the cell identically to the native pheromone. However, this analog is biologically inactive as a mating pheromone. From this result and the observation that *a* cell metabolites or trypsin-digested rhodotorucine *A* (data not shown) had no biological activity for sexual differentiation, we assume that metabolites alone are not sufficient but that both the metabolic reaction and its products are required for the signaling process. Since one of the metabolites, the amino-terminal octapeptide segment, produced by the analog is identical to that of the native pheromone, a carboxy-terminal isoprenyl tripeptide from the native pheromone may be important for the signaling process. The competitive inhibitory effect of the analog against the native pheromone suggests that an oxidized derivative of tripeptide not only is inactive as a signaling substance but also quantitatively competes against an effective signaling process by the native pheromone.

Although a majority of the mating pheromone administered to the suspension of *a* cells was recovered in the cell suspension medium as two proteolytic segments, a small but significant amount of the radioactivity remained associated with the cell. From the data shown in Fig. 4, the number of cell-associated, ^{125}I-labeled molecules after 1 h of incubation at 28°C was estimated to be approximately 200 per cell in the presence of saturating amounts of the pheromone. Chemical and topological characterization of the cell-associated molecules should be important for the elucidation of processes for signal transmission. Because of technical difficulties in preparing *R. toruloides* protoplasts and in isolating small amounts of the radioactive compounds associated with the cell, these problems remained to be solved. Mating pheromone associated with the cell at 28°C incubation (Fig. 4) may represent pheromone incorporated through the membrane, and rapid loss of the bound radioactivity at 0°C (Fig. 4) may be due to hydrolysis by the pheromone metabolic activity of cell-associated pheromone before it is transported through the membrane, due to a decreased rate of penetration at this low temperature.

It has been reported from several laboratories that the α-factor of *S. cerevisiae* is metabolized by proteolysis by the target cell (4, 6, 11, 19). However, the biological effect of the mating pheromone is potentiated by inhibition of α-factor metabolism (4). It is suggested that α-factor metabolism is required for the detoxication of α-factor, i.e., for recovery from α-factor-induced G1 arrest of the cell cycle (4, 6, 19). The apparent difference in the role of pheromone metabolism in the two organisms might be explained by differences in the chemical structure of the mating pheromone or morphological differences in the sexual differentiation process in the two organisms. Schmoo formation induced by α-factor in **a** cells of *S. cerevisiae* seems to be related to a morphological change which occurs as a result of cell cycle arrest in G1 at the start event of the cell division cycle, since *cdc* mutants defective at this point of the cell cycle (class I start mutants) result in a similar morphological change (15). In contrast, elongation of a mating tube in *R. toruloides* is directed by the concentration gradient of rhodotorucine *A* (S. Takasaki, unpublished data), resulting in a remarkable oriented growth of the mating tube on the solid medium, toward cells of the opposite mating type (1). Pheromone metabolism might be involved in the elongation of the mating tube and in the recognition of the concentration gradient of the mating pheromone.

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