Cytochemical Observations on the Localization of Sulphydryl Groups in Budding Yeast Cells and in the Phialides of *Penicillium notatum* Westling during Conidiation

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SUMMARY

Fluorescence and chromogenic cytochemistry confirmed the presence of –SH groups in the walls of *Candida albicans* and *C. utilis*, predominantly at budding sites. They were absent from pseudomycelia of *C. albicans* and the walls of the mycelium and the hyphal tips of *Penicillium notatum* but present in the walls of the tips of the phialides of *P. notatum*. Autoradiography after treatment with tritiated SH-blocking agents confirmed these findings.

INTRODUCTION

Certain yeast species have sulphydryl compounds as structural components of the walls with higher concentrations at sites of bud formation (Nickerson & Falcone, 1958). A conversion of disulphide (–SS–) to sulphydryl (–SH) groups in the protein of the wall may allow re-orientation of the wall fibrils and so permit plastic deformation and distension of the wall for bud formation. The walls of mycelial fungi contain relatively few –SH groups (Robson & Stockley, 1962).

Robson & Stockley (1962) fulfilled the prediction of Nickerson & Falcone (1958) that suitable cytochemical techniques might reveal –SH groups at budding sites in yeasts when they demonstrated such groups at budding sites in *Eremothecium ashbyi* and *Candida albicans* by using tritiated phenyl mercuric chloride.

The walls of the phialide tips in *Penicillium notatum* cover a zone which undergoes rapid division during sporulation. Such a zone might be analogous to the sites of bud formation in yeasts and spore formation might need high concentrations of –SH groups at such sites. A number of recent developments in histochemistry have provided highly sensitive and specific chromogenic reagents for protein bound –SH groups and these have been used in the current work to re-examine the budding process in yeasts, to seek confirmation of results by autoradiographic techniques and to examine the position in *P. notatum*.

METHODS

Organisms. *Penicillium notatum* Westling, *Candida albicans* (Robin) Berkhout and *C. utilis* (Henneb.) Lodder & Kreger-van Rij, designated E9, E25 and E135 respectively in the culture collection of the Exeter Botany Department, were used. *Penicillium notatum* was grown on coverslips for 3 days at 25° on discs of malt extract agar and the

yeasts were inoculated from stationary phase liquid cultures into 2% malt extract liquid medium and grown for 18 hr at 25° in shaken vessels.

Preparation of reagents. (a) *N*-(4-hydroxy-1-naphthyl) *iso*-maleimide (HNI) was prepared according to the method of Tsou, Barnett & Seligman (1955). (b) *p*-*N,N*-dimethylaminophenylmercuric acetate was made by the method of Lillie (1965). (c) Tritiated phenyl mercuric chloride was prepared in very low yield by the method of Robson & Stockley (1962); subsequently the following procedure was devised: 0.25 ml. HCl (sp.gr. 1.18), 0.3 ml. H₂O and 25 mg. (10 mc.) ring-tritiated aniline (Radiochemical Centre, Amersham) were stirred at room temperature for 10 min. and then cooled to 0°. 0.5 ml. (0.17 g.) NaNO₂ was then added followed by vigorous stirring for ½ hr at 0°. 0.42 g. HgCl₂ was dissolved in 0.2 ml. HCl (sp.gr. 1.18), 1 ml. H₂O was added and this mixture was added dropwise to the stirred diazotate at 0° over 5 min. The mixture was stirred for a further ½ hr at 0°, the addition compound was filtered off in the cold and washed with iced water. The wet compound was then suspended in 5 ml. acetone at 20° and small amounts of an active copper powder were added over ½ hr with stirring until effervescence ceased. Stirring was continued for 20 min. and 5 ml. H₂O was added. The suspension was filtered and the precipitate washed and extracted with boiling xylene and recrystallized twice from this solvent. Yield 40 mg.

Microscopy. A Leitz 'Ortholux' microscope equipped with a transmitted light condenser and an incident light attachment was illuminated with a tungsten lamp or a 250 w high-pressure mercury vapour lamp situated in either the incident or transmitted light positions for white light and fluorescence microscopy. Unfixed material was sometimes examined but generally fixed materials were used after being coated with 0.5% collodion before staining.

Chromogenic reactions for sulphydryl groups. (a) The dihydroxy-dinaphthyl-disulphide (DDD) procedure of Barnett & Seligman (1952) was done on material of *Penicillium notatum*, *Candida albicans* and *C. utilis* which had been freeze-dried and subsequently fixed for 2 to 3 hr in 1% acetic acid in absolute alcohol. Controls blocked with iodoacetate, *N*-ethyl maleimide (NEM) or phenyl mercuric chloride and others incubated with the coupling agents but no DDD were prepared. Also disulphides in the material were reduced with thioglycollate before applying the full DDD reaction with appropriate controls. (b) The *N*-(4-hydroxy-1-naphthyl) *iso*-maleimide method with coupling with Fast blue B salt (G. T. Gurr) or Fast blue RR salt (G. T. Gurr) according to Pearse (1969) was applied to Carnoy fixed material and material freeze-dried and fixed in 1% acetic acid in absolute alcohol for 2 to 3 hr and stored in absolute alcohol. Material of *P. notatum* and the two yeasts was stained for 24 hr and then examined by visible light and by transmitted light fluorescence microscopy using Leitz UG 1 + BG 38 filters and suppressor filters. In controls HNI was omitted from the incubation medium, the coupling stage was omitted from the full reaction, and iodoacetate or NEM was used to block -SH groups prior to incubation in HNI. (c) The performic acid + Alcian blue method of Adams & Sloper (1956), using performic acid prepared according to Pearse (1969), was applied to material fixed for 12 hr in Baker's formol + calcium fixative. Since this method stains both -SS- and -SH groups other material was reduced with thioglycollate and the original and newly formed -SH groups were blocked by iodoacetate. The differences between this material, that stained by the normal method, and that stained normally after iodoacetate blocking

indicated the abundance of -SS-groups in the original material. (d) Material was freeze-substituted or freeze-dried and fixed in 1% acetic acid in absolute alcohol for 2 to 3 hr and subjected to the Mercury orange staining procedure of Bennett & Watts (1958) for 1 to 3 days at 20°. A saturated solution of 1-(4-chloromercuriphenylazo)-2-naphthol, known as Mercury orange (Koch-Light Laboratories Ltd.), in dimethyl-formamide was used. Before it was stained control material was treated with a saturated solution of phenyl mercuric chloride in *n*-butanol for 48 to 72 hr or with NEM. The mercaptide azo coupling reaction for sulphydryl groups of Lillie & Glenner (Lillie, 1965) was also used, with incubation in 0.7% *p*-*N,N*-dimethylaminophenylmercuric acetate in 99% isopropanol at 25° for 24 to 48 hr. (e) Fresh and freeze-dried material was subjected to the NEM+salicyloyl hydrazide+zinc method of Stoward (1963). Preparations were brought to water, incubated in NEM for 4 hr at 37°, treated with salicyloyl hydrazide (Sigma Chemical Co.) for 20 to 120 min., rinsed in 0.05% sodium pentacyano-amine ferroate to extract excess hydrazide, and then examined by incident and transmitted light fluorescence microscopy using UG 1 + BG 38 filters and suppressor filters. 5% potassium alum or 0.1% Solochrome black in 5% potassium alum were used as counterstains (Stoward, 1967).

Autoradiographic methods for sulphydryl groups. (a) Preparations of *Penicillium notatum* and yeast smears were freeze-dried fixed in 1% acetic acid in absolute alcohol for 2 to 3 hr, taken into dioxan, incubated in tritiated phenyl mercuric chloride (0.001M) for 72 hr, washed in two changes of acetone (15 min. each), coated with stripping film (Kodak AR 10), and exposed for 1 month at 4° before development in Kodak D 19 b developer. The autoradiographs were dehydrated, cleared in xylene and mounted in 'Euparal'. (b) Iodoacetic acid-2-T (Radiochemical Centre, Amersham; specific activity 0.68 mc./mg.) was titrated to pH 8.0 with sodium hydroxide and made 0.1M with respect to sodium iodoacetate-2-T. Material grown on slides was freeze-dried, fixed, coated with collodion, brought to water and incubated in 0.1 M-tritiated iodoacetate for 20 hr at 37°. It was washed three times in distilled water, coated with stripping film, exposed for 2 to 3 weeks, developed and permanently mounted.

Sulphydryl blocking and disulphide reduction reactions for protein end-groups. (a) Iodoacetate block. Collodion-coated preparations were treated for 20 hr at 37° with 0.1M-sodium iodoacetate at pH 8.0. (b) Maleimide block. Material was treated for 4 hr at 37° with 0.1M-*N*-ethyl maleimide in 0.1M-phosphate buffer pH 7.4, and washed in 1% acetic acid and water. (c) Mercaptide block. Collodion-coated material was incubated at 20° for at least 72 hr in 0.001M-phenyl mercuric chloride in *n*-propanol. (d) Thioglycollate reduction. Collodion-coated material was incubated for 4 hr at 37° in freshly prepared 0.5M thioglycollic acid titrated to pH 8.0 with 0.1N-NaOH, washed in water, rinsed in 1% acetic acid and washed again in water.

RESULTS

Chromophoric sulphydryl staining

In Penicillium notatum. The DDD method incorporating a number of diazotates strongly stained the tips of the phialides and the spores. The walls of the mycelium did not stain, but the cytoplasm near the hyphal tips did. Bis-coupling with Fast blue B salt stained the cytoplasm of both the mycelium and phialides pink and areas of

higher sulphhydryl concentrations such as the nuclei, spores and walls at the phialide tips blue. When the monocoupling diazotate Fast black K salt was used the walls of the phialide tips and the spores stained intense black (Pl. 1, fig. 1). Staining was prevented by blocking with NEM and iodoacetate (Pl. 1, fig. 2) but only partially prevented with phenyl mercuric chloride. Control material incubated without DDD stained pale brown near the phialide tips when Fast blue salt B was used, but not with Fast black K salt or Fast red RC salt. Preparations reduced with thioglycollate stained similarly to unreduced ones, indicating that staining due to -SS-groups was negligible by this method.

The HNI procedure stained the cytoplasm a very pale purple colour by visible light, but fluorescence microscopy in which a range of diazotates was used revealed intense red staining in the nuclei, spores, cytoplasm of the hyphal tip and the walls at the tips of the phialides (Pl. 1, fig. 3); walls of the mycelium did not stain. Prior incubation of material in NEM or iodoacetate abolished the red fluorescence and replaced it by a dull general autofluorescence.

The performic acid + Alcian blue method stained nuclei and spores and especially hyphal tips and the walls near the tips of the phialides (Pl. 1, fig. 4). Preparations reduced with thioglycollate and blocked with iodoacetate before staining showed negligible staining (Pl. 1, fig. 5). A low level of staining in materials that had been blocked with iodoacetate beforehand, suggested some staining was due to -SS-groups.

The Mercury orange method of Bennett & Watts (1958) did not stain material even after several days' incubation. The *p*-*N,N*-dimethylaminophenylmercuric acetate procedure stained walls red at the phialide tips, but not elsewhere in the mycelium, when coupled with freshly prepared diazosafranin (Pl. 1, fig. 6).

The NEM + salicyloyl hydrazide + zinc method gave only a pale green stain with a general distribution and was not improved by counterstaining with alum or Solochrome black in alum.

In yeasts. Although the DDD, HNI and Lillie & Glenner's mercaptide methods all stained nuclei in *Candida albicans* and *C. utilis* by visible light methods, none of these methods was sensitive enough to detect sulphhydryl groups in the wall. The Alcian blue method stained both nuclei and cell walls, and blocking and reduction reactions had effects similar to those observed in *Penicillium notatum*. Nuclei and cell walls of the yeast phases of *C. albicans* and *C. utilis* stained by the HNI method fluoresced a brilliant red, but there was only very slight fluorescence in the walls of the pseudomycelium of *C. albicans* (Pl. 1, fig. 7). Observations were not made on the mycelial state of *C. utilis* because such growth was not supported by the media routinely used.

The NEM + salicyloyl hydrazide + zinc procedure gave a pale green fluorescence which was almost indistinguishable from the autofluorescence of the materials.

Autoradiographic localization of sulphhydryl groups

Preliminary experiments with 0.001 M-phenyl mercuric chloride to block cellular -SH groups before the DDD reaction showed that blocking was incomplete with *Penicillium notatum* and the yeasts even after 72 hr incubation in the blocking agent. Because it was impracticable to incubate longer without losing material from the slides 72 hr incubation was adopted for all experiments using tritiated phenyl mercuric chloride.

Autoradiographs of *Penicillium notatum* treated with tritiated phenyl mercuric

chloride showed grain concentrations around the spores and developing phialides but not in the walls of the mycelium. Preparations treated with tritiated iodoacetate showed concentrations of grains at the walls of the phialide tips but not in the spores (Pl. I, fig. 8), and none in the walls of the mycelium.

Autoradiographs of yeast-like cells of *Candida albicans* and *C. utilis* treated with tritiated phenyl mercuric chloride showed grains in the walls especially at sites of bud formation and bud scars (Pl. I, fig. 9). Similar results were obtained after treatment with tritiated iodoacetate. The concentration of grains was much less near the walls of pseudomycelium of *C. albicans*.

DISCUSSION

The role of protein-bound sulphydryl groups in maintaining the plastic condition of the cell wall in budding yeasts is now well established (Nickerson & Falcone, 1958; Nickerson, Falcone & Kessler, 1961), although McClary & Bowers (1965) challenged this view and considered that the wall neither distends nor disrupts during budding.

Robson & Stockley (1962) provided convincing autoradiographic evidence of -SH groups at budding sites in *Candida* and *Eremothecium*. The present work confirms these autoradiographic findings and supplies additional cytochemical evidence of the presence of -SH compounds in the walls of yeast cells of *Candida albicans* and *C. utilis* and their absence from the pseudomycelium of the former.

It is not known if -SH groups serve a similar function in maintaining plasticity in hyphal tips of mycelial fungi. The present work supports the findings of Zalokar (1959) in showing a higher concentration of cytoplasmic -SH groups near the hyphal tips than elsewhere in the mycelium. However, the observations of Middlebrook & Preston (1952) with *Phycomyces* suggest that there is no need to postulate a mechanism for maintaining sulphur in the reduced state as -SH groups in order to render the walls plastic because the orientation of the wall fibrils satisfies the requirements of extension growth and branching. The present work failed to show -SH groups in any part of the wall of *Penicillium notatum* besides the tips of the phialides. Such a localization in areas that proliferate cells rapidly during sporulation in a manner analogous to budding in yeasts supports the hypothesis that sporulation in *P. notatum* may have physiological processes in common with budding in yeasts. However, this localization does not necessarily implicate such compounds in sporulation. Biochemical analysis and electron microscope cytochemistry of the walls of developing phialides of *P. notatum* are necessary to provide further evidence.

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EXPLANATION OF PLATE

Cytochemical localization of —SH groups in *Penicillium notatum*, *Candida albicans* and *Candida utilis*.

Fig. 1. Sulphydryl groups in the walls of the phialide tips of *P. notatum* stained by the DDD procedure, coupled with Fast black K salt. $\times 1350$.

Fig. 2. *P. notatum* stained as in fig. 1, but with prior blocking in iodoacetate. $\times 1350$.

Fig. 3. Sulphydryl staining in *P. notatum* by the HNI reaction and observed by fluorescence microscopy. Note strong fluorescence in the spores and in the walls of the phialide tips (red in the original). $\times 1350$.

Fig. 4. Alcian blue staining of —SS— and —SH groups in *P. notatum*. Strongest reaction in the spores and the walls of the phialide tips. $\times 1350$.

Fig. 5. As fig. 4 but with thioglycollate reduction and iodoacetate blocking before staining; negligible staining. $\times 1350$.

Fig. 6. Sulphydryl staining of *P. notatum* by Lillie & Glenner's mercaptide method. Staining in the spore walls and the walls of the phialide tips. $\times 1350$.

Fig. 7. Cell wall staining of sulphydryl groups in *C. albicans* by the HNI fluorescence method showing increased staining at some sites in the wall. Note very pale cytoplasmic staining in the pseudomycelium with negligible wall staining. Coupled with Fast blue RR salt. $\times 1500$.

Fig. 8. Arrow 1 indicates autoradiographic localization of —SH groups in the phialide tip of *P. notatum* with tritiated phenyl mercuric chloride as the blocking agent. $\times 1350$.

Fig. 9. Autoradiographic localization of —SH groups in *C. utilis* with tritiated iodoacetate as the blocking agent. Note highest concentrations of grains at sites of budding and bud scars. $\times 2000$.

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