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Identification of Protein Disulfide Reductase as a Cellular Division Enzyme in Yeasts

Previous work in this laboratory (*1*) has shown that divisionless mutant 806 of *Candida albicans* is genetically blocked at a reductive reaction in such a manner that metabolically generated hydrogen is "spilled over" in quantity, during growth, for nonspecific reductions. (Added dyes, and tetrazolium compounds, with redox potentials as low as $E_0' = -0.150$ v are readily reduced by growing cells of the mutant but not by the parent strain). This "waste" of reductive capacity by the mutant is not at the expense of demands of hydrogen acceptors participating in syntheses essential for growth, since mutant and normal strains synthesize cell mass at approximately the same rate; nor is it at the expense of respiration, since the mutant reduces oxygen even more rapidly than does the normal strain (*2*).

It is possible environmentally to induce filamentation and dye reduction in the normal strain by the incorporation of a powerful metal-chelating agent in the culture medium. Based on these facts, a biochemical lesion underlying the morphological alteration has been postulated to involve a flavoprotein locus, which has been converted to a diaphorase (causing nonspecific dye reductions) in the filamentous form, but which is presumably a metallo-flavoprotein in the normal strain and catalyzes the reduction of some hydrogen acceptor participating in cellular division.

The existence of a protein (containing 2.1 percent sulfur) bound to the mannan component of the cell wall of baker's yeast has recently been demonstrated (*3*). Enzymatic reduction of disulfide linkages in this protein was achieved by the use of cell-free particulate preparations from baker's yeast (*4*). Polysaccharide-protein complexes (containing about 2 percent sulfur) have also been found in the clean cell walls of both normal and filamentous strains of the yeast *C. albicans* (*5*). We wish to report that mitochondrial particulates obtained from the normal strain of *C. albicans* show powerful protein disulfide reductase activity on cell-wall protein, whereas similar preparations from the divisionless (filamentous) mutant possess such activity only to a very slight extent. The data presented in this paper permit identification of the "hydrogen acceptor participating in division" as the disulfide bond of the cell-wall mannan-protein component. Furthermore, the protein disulfide reductase that catalyzes this reduction may be termed a "division enzyme" and is the first such catalyst to be identified.

Normal strain 582 of *C. albicans* (ATCC No. 10261) and filamentous mutant strain 806 (ATCC No. 10259) were grown in a glucose, ammonium sulfate, biotin, salts medium with continuous agitation at 28°C for 48 hours. Batches of 20 lit were grown in 2-lit flasks (0.5 lit per flask) on a large reciprocating shaker to obtain sufficient cells for preparation of isolated clean cells walls by the methods previously described (*3*). Batches of 4 lit were grown in 250-ml flasks (100 ml per flask) on a rotatory shaker to obtain cells from which mitochondrial particulates were isolated, as was previously described (*4*). Mannan-protein was solubilized from isolated clean cell walls (*3*), and sulfhydryl groups of the protein were oxidized with ferricyanide. The resulting oxidized cell-wall protein was incubated with active or boiled mitochondria isolated from both strains of yeast (*4*). After incubation, the reaction mixture was centrifuged at high speed to remove particulates. The —SH content of the protein in solution was assayed spectrophotometrically by mercaptide formation with *p*-chloromercuribenzoate by the method of Boyer (*6*).

As is shown in Table 1, mitochondrial particulates isolated from the normal strain of *C. albicans* exhibit vigorous protein disulfide reductase activity against the cell-wall protein obtained from this strain and from the mutant. In contrast, the mutant exhibits only slight enzymatic reduction of —S—S— linkages of its own cell-wall protein. The relative activities, per unit density of mitochondrial suspension, for normal versus mutant strain were 24.4 to 1.0. Whereas mitochondria from the normal strain vigorously reduced disulfide of the mutant cell wall (7.3 times faster than the mutant itself), mitochondria from the mutant strain were completely lacking in ability to reduce —S—S— of the normal cell wall. Enzymatic reduction of disulfide covalent bonds in mannan-pseudokeratin, a major structural component of the cell wall of yeasts, is thus seen to be a reaction essential for cellular division of yeasts.

It is not possible here to consider the physical consequences of formation and breakage of covalent bonds between the molecular fibrils that make up the fabrics that constitute the cell wall of yeasts. However, it seems important to mention two aspects of this attempt to analyze the molecular bases of this area of cellular morphology. Examination of individual frames of time-lapse photographs (darkfield microscope) of budding yeast (*7*) reveals, in a most striking manner, that a bud-initial arises as a "blowout" from the mother cell. Localized enzymatic reduction of protein disulfide may operate at this stage of the division process to convert a portion of

Table 1. Protein disulfide reductase activity in normal and divisionless strains of *Candida albicans*.

Reaction system*	Mercaptide formation† (optical density at 255 mμ)	
	Enzyme system from normal yeast	*Enzyme system from divisionless mutant*
Oxidized cell-wall protein from normal yeast	1.156	1.156
Mitochondrial particulates	0.520	0.790
	1.676	1.946
Cell-wall protein + particulates‡	3.800	1.880
Oxidized cell-wall protein from filamentous mutant	0.766	0.766
Mitochondrial particulates	0.520	0.790
	1.286	1.556
Cell-wall protein + particulates‡	1.920	1.730

* The components indicated were added to the following basal mixture and incubated at 37°C for 2 hours: sodium succinate, 10 mg; ethanol, 4.5 mg; liver coenzyme concentrate (Armour) 50 μg; and 0.02*M* phosphate buffer, *p*H 7.0; reaction volume 3.5 ml. Where indicated, mannan-protein from 12 mg cell wall, solubilized as described (*3*), and 0.5 ml of mitochondrial particulate suspension (in 8.5 percent sucrose) were added.

† For determination of sulfhydryl content of protein, 2.0-ml samples were taken from the incubated mixtures and added to 1.0 ml of 0.3M acetate buffer, *p*H 4.6, and 0.5 ml 1.2×10^{-4}M *p*-chloromercuribenzoate (assayed spectrophotometrically at 234 mμ in 0.1M acetate buffer, *p*H 4.6, according to the method of Boyer, *6*). Mercaptide formation was allowed to proceed for 90 minutes at 37°C and then determined at 255 mμ.

‡ Values for controls using heated mitochondria were not greater than the sum of the constituents.

the "vulcanized" cross-linked wall fabric into a form capable of plastic deformation.

As is revealed by the electron microscope, polymer formation in the direction of growth develops as a densely intermeshed fibrillar network appropriate to an expanding prolate spheroid. It is important to note that covalent bonding between fibrils of the wall components that are not linearly ordered very likely serves to increase the modulus of elasticity in proportion to the number of covalent bonds (*8*). This feature of the wall fabric is undoubtedly essential for the expanding spheroid. The "ring type" of ordered fibrillar structure observed in "bud scars" serves to constrict the base of the bud in a plane normal to the direction of growth of the bud. This arrangement of fibrils is beautifully illustrated in electron microscope photographs of the yeast glucan layer by Houwink and Kreger (*9*). It is in the ordering of these fibrils that covalent bonding is believed to interfere. This may be a second stage in the division process during which maintenance of a sulfhydryl condition in a structural polymer is temporarily essential. Covalent (—S—S—) bonds might be formed, however, after the ordered arrangement has been achieved.

The molecular events in cellular division that have been uncovered thus far bring into fold many scattered, apparently unrelated, observations of the environmental control of cellular division. It is now intelligible, for example, that sulfhydryl substances applied externally to growing, but nondividing, cells might induce division in such cells (*10*). It is becoming increasingly clear that a cell possesses a variety of systems, each with its degree of specificity, for maintaining functional —SH groups. Those employed in growth may be operative while a disulfide reductase essential for division may have failed. In fact, this exact situation is met in the filamentous strain of *C. albicans,* which possesses both an active glutathione reductase and cystine reductase (*11*) but is deficient in a protein disulfide reductase.

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"Permanent" Alteration of Behavior in Mice by Chemical and Psychological Means

We wish to report a behavioral response in mice which may be induced by both chemical and psychological means and which responds in some measure to drugs useful in mental illness. We had previously demonstrated (*1*) that fish exposed to LSD (lysergic acid diethylamide), upon return to a normal environment, exhibited an unusual and characteristic behavior pattern, and that this pattern could be rendered "permanent" by appropriate chemical treatment of the fish. In an attempt to extend this type of study to mice, we first searched for alterations in behavior that might be consistently attributed to treatment with LSD. We found that the behavior pattern described by Woolley (*2*) could be confirmed and that it was reasonably reproducible, but in the meantime Lars Flataker had pointed out to us a behavior response that was easier to determine.

The response consists of a rapid and violent head shaking when any area about the back of the head is touched very lightly with a small stick or pencil point. The head-twitch response does not occur in normal mice, and with a little experience the response is easy to detect. It is only rarely that one is uncertain whether a particular animal possesses the head twitch or not, and usually, if such an animal is followed, a clear-cut answer is found on the subsequent days. Independent observations by different workers are remarkably consistent, so that this provided a suitable tool for the behavioral studies.

If mice are injected intravenously with from 5 to 100 μg LSD (0.25 to 5 mg/kg), this characteristic head shake response appears in from 5 to 10 minutes and lasts for intervals of from 10 minutes to 2 hours, the length of time it persists being roughly proportional to the amount of LSD used. A variety of other substances (for example, mescaline, yohimbine, serotonin) do not elicit this response. The response is consistently reproducible in 90 to 100 percent of the animals injected and seems to occur in several strains of mice tested.

In an effort to render the head-twitch response "permanent," mice were injected intravenously with 30 μg indole (1.5 mg/kg), followed almost immediately by 30 μg LSD. All such mice showed the head-twitch response, but, in the majority of the cases, it subsided in 1 to 2 hours, and the mice were subsequently normal. However, in from 5 to 30 percent of the mice so treated (housed in groups of 10 each) the response remained for periods as long as a week, and in some cases for a few months. We have apparently therefore produced a "permanent" alteration in a particular aspect of mouse behavior by chemical treatment, in analogy to the alterations in fish (*1*), but such "chemical imprinting" is apparently more difficult in the mouse. Injection of either indole alone or saline produced no response, while injection of LSD alone produced only the usual temporary response.

It was also found that the identical response was produced in a larger percentage of mice by solitary confinement. The mice were placed in separate cages (one mouse to a cage) in which they had access to light, sound, and so forth, but were unable to see any other mice. Under these circumstances roughly 30 percent of the mice developed the head-shake response in 2 days (this percentage varied from 30 to 80 percent, depending on the strain of mouse employed). If such mice were kept in solitary confinement for 3 weeks (2 weeks is not adequate) and were then returned to groups (8 to 10 in a group), generally about 80 percent retained the response for weeks and even months. One has, under these circumstances, apparently rendered the response permanently "implanted." If such mice, in which the head-shake response has been permanently established, are treated with reserpine (5 mg/kg – p.o. for 3 days) about half become symptom free in 2 to 3 days and remain in this state as long as reserpine is supplied. When the reserpine is withdrawn, the head-shake response gradually reappears, even though the mice are kept in groups.

About another third of the mice do not develop the symptom until they have been kept in solitary confinement for at least a week. When these are grouped after 3 weeks of solitary confinement, the response is retained in most of the animals but is slowly lost with time. If such animals are given reserpine, most of them promptly lose the