Ultraviolet Micrography of Penetration of Exogenous Cytochrome c into the Yeast Cell

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Candida utilis and Saccharomyces cerevisiae in water suspension were found to be very sensitive to exogenous cytochrome c. The protein was taken up by the cells, and the viable count was reduced to a few per cent of the initial value. Micrography at 405 nm revealed penetration of cytochrome c into the interior of the cell. The cytoplasmic membrane lost its capacity to retain intracellular constituents, and ultraviolet-absorbing compounds were released into the medium. When budding cells were subjected to treatment with cytochrome c, the mother cells were found to be more susceptible than the buds. Phosphate buffer protected the cells and spheroplasts against cytochrome c.

We have reported that yeast cells in water suspension or in medium of low ionic strength are injured by various basic proteins such as ribonuclease, protamine, lysozyme, bovine serum albumin, myoglobin, and cytochrome c (1, 2, 8). The cellular membrane loses its retentiveness for cytoplasmic solutes of small molecular weight, the intracellular organization is deranged, and the cells die rapidly (7, 14).

The mode of action of these proteins is as yet unexplained. It appears that they penetrate the cell wall and act directly on the membrane. The possibility of an enzymatic effect on the membrane is excluded by the fact that several of the effective proteins are without known enzyme activity. A physical interaction between the proteins and the yeast cell membrane appears to be involved.

The present experiments were carried out to determine (i) the extent of the action of the injurious protein and whether it acts only on the cell wall or membrane or on other cell organelles as well; (ii) the distribution of the protein within the cell; and (iii) the nature of the lesions in the membrane and whether they are temporary or permanent.

Cytochrome c from horse heart was selected as the protein for this investigation because of its characteristic color which facilitates microscopy. The quantities used were several orders of magnitude higher than the concentration of endogenous cytochromes; hence no interference from the intracellular material had to be anticipated. It should be emphasized that the action of the exogenous cytochrome c is unrelated to its biological function in the respiratory system of the cells examined.

MATERIALS AND METHODS

Candida utilis (ATCC 9950) and Saccharomyces cerevisiae (ATCC 7752) were employed. The composition of the medium, which contained ammonium sulfate, glucose, and salts, has been given earlier (8). For the culture of S. cerevisiae, a supplement of 0.5% yeast extract (Difco) was used. Occasionally, a supplement of sulfur amino acids was used to enrich the cells with S-adenosylmethionine (9, 10). Unless noted otherwise, the cells were harvested after 40 to 48 hr at 30°C, washed twice with distilled water, and stored at 2 to 4°C. Viable counts were determined by plating on wort agar (Difco) in triplicate. The initial counts of washed and compacted cells were 0.8 × 10⁹ to 10¹⁰ organisms per g. For experiments in isotonic medium, 1.0 M mannitol or sorbitol was used; the latter offers the advantage of higher solubility when solutions or cell suspensions are stored at low temperature. For osmotic adjustment, the cells were washed with isotonic medium if the subsequent experiments were carried out in isotonic solutions. All cell weights given in the experiments pertain to wet compacted yeast.

Spheroplasts were obtained from C. utilis by the action of purified Helix pomatia enzymes (Glusulase, Endo Laboratories, Garden City, N.Y.). Cells that had been cultured for 40 to 48 hr in medium supplemented with sulfur amino acids were used (11, 13). They respond more readily to the wall-digesting enzymes than cells from non-supplemented medium. A 50-μl amount of cells and 50 μl of Glusulase per ml of 1.0 M sorbitol, which contained 0.05 M tris-(hydroxymethyl)aminomethane-citrate buffer (pH 6.2), were incubated at 25°C. The change of the ovoid
cells into spherical protoplasts (spheroplasts) reached a maximum after 2 hr; uniform microscopic appearance and bursting of samples after dilution with water indicated completion of the process. The spheroplasts were washed with 1.0 M sorbitol.

For the experiments, a final concentration of 2.0 mg of moist yeast cells per ml was used throughout; the concentrations of cytochrome c varied from 25 to 230 μg/ml. The incubation time was 30 min at 30 C. After centrifugation, residual cytochrome c was measured in the supernatant fluid at 408 nm, and the release of cytoplasmic material was measured at 260 nm. Deviations from this regimen are noted in connection with the experiments.

Cytochrome c from horse heart (type III, Sigma Chemical Co., St. Louis, Mo.) was used; other preparations were found to be as suitable, provided they were free from salts.

Measurement of the release of cytoplasmic constituents absorbing at 260 nm was used as an indicator of injury to the cytoplasmic membranes. Alternatively, the release of amino acids (14) or the release of organic and inorganic phosphates (8) could have been used, but these methods were found to be more time-consuming. For quantitative assay of the uptake or release of cytochrome c, the Soret band of the compound (3) at 408 nm (A_m = 0.9 × 10^6) was employed. If nucleic acids and related compounds were measured at 260 nm in the solution that contained cytochrome c, a correction was made for the absorbance of cytochrome c at that wavelength; in most preparations, this amounted to 20% of the absorbance in the Soret region. The usefulness of S-adenosylmethionine (A_m at 260 nm = 1.5 × 10^6) as an optical marker and contrast substance is obvious from the fact that, because of its high concentration, its absorbance at 260 nm in enriched cells exceeds the total absorbance of deoxyribonucleic acid, ribonucleic acid, and all other cellular substances that also absorb at 260 nm (12).

Cytological studies were carried out by ultraviolet (UV) micrography at wavelengths near the absorbance maxima used in spectrophotometry. Wavelengths of 405 and 265 nm were used, rather than 408 and 260 nm, because of the characteristic line emission of the mercury lamp employed as the UV source. For reference, pictures at 297 nm were included; at this wavelength the absorbance of cellular solutes is low, and details that are masked by high absorbance at the other wavelengths can be seen. A detailed account of our ultraviolet micrographic methods is given elsewhere (5, 6).

RESULTS

When a solution of cytochrome c was mixed with a suspension of yeast in water, the cytochrome was rapidly taken up by the cells and UV-absorbing cytoplasmic constituents were released into the medium (Fig. 1). The effect of the cytochrome c (Fig. 1) was very rapid, but to assure completeness of action longer incubation periods were used. A 15-min period was usually sufficient. The cells may bind as much as one-third to one-half of their own dry weight. In contrast to this, there was no effect on S. cerevisiae or C. utilis when cytochrome c (200 μg/ml) was added to the culture medium; the cells were fully protected by the presence of salts in the medium. After 40 hr at 30 C, the usual quantity of cells (3 g/100 ml) was harvested, and all of the cytochrome c was present in the supernatant fluid, as judged by spectrophotometry at 408 nm. The washed cells from these cultures, however, showed the same sensitivity toward cytochrome c as those from cultures without the protein. As a preliminary to cytological studies, the optimal conditions for the action of cytochrome c on yeast cells were examined.

There was little variation in the uptake of cytochrome c and in the release of UV-absorbing cytoplasmic constituents by washed cells having cultural ages of 4, 7, 16, and 40 hr. Microscopy, however, revealed individual differences among cells (see below). Earlier experiments (14) had suggested that cations interfere with the action of cytochrome c. A more detailed examination revealed that the uptake of cytochrome c was...
**Table 1. Reversibility of cytochrome c uptake by yeast cells**

<table>
<thead>
<tr>
<th>Eluting agent in water or 1.0 M sorbitol</th>
<th>Release of cytochrome c from</th>
<th>C. utilis</th>
<th>S. cerevisiae</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>In water</td>
<td>In 1.0 M sorbitol</td>
<td>In water</td>
</tr>
<tr>
<td>Water</td>
<td>5.8</td>
<td>2.6</td>
<td>7.6</td>
</tr>
<tr>
<td>1.0 M Sorbitol</td>
<td>68</td>
<td>83</td>
<td>54</td>
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<tr>
<td>0.1 M KCl</td>
<td>79</td>
<td>93</td>
<td>70</td>
</tr>
<tr>
<td>0.5 M KCl</td>
<td>75</td>
<td>86</td>
<td>46</td>
</tr>
<tr>
<td>0.1 M Potassium phosphate, pH 6.1</td>
<td>73</td>
<td>92</td>
<td>60</td>
</tr>
<tr>
<td>0.5 M Potassium phosphate, pH 6.1</td>
<td>87</td>
<td>92</td>
<td>49</td>
</tr>
<tr>
<td>0.1 M K$_2$HPO$_4$</td>
<td>90</td>
<td>91</td>
<td>63</td>
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<tr>
<td>0.5 M K$_2$HPO$_4$</td>
<td>73</td>
<td>77</td>
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* The cells, 2.0 mg per ml of water or 1.0 M sorbitol, were saturated with cytochrome c (200 µg/ml). After 30 min at 30°C, they were centrifuged, and the residual amount of cytochrome c in the supernatant fluid was determined by spectrophotometry. The average uptake of cytochrome c by the cells was 87% of the quantity added. The cells were washed in water or 1.0 M sorbitol and suspended in the solutions listed above. After 30 min at 30°C, the quantity of cytochrome c released was measured in the supernatant fluid by spectrophotometry at 408 nm. The percentages given are based on the initial uptake.

Incomplete, and the release of UV-absorbing constituents was impeded at potassium ion concentrations higher than 4 mM and at pH values above 5. To eliminate the cation effect of buffer solutions, acetic acid alone was used to vary the pH of the yeast suspensions. At a level of 0.5 mM, acetic acid lowered the pH value to 5.5 without interfering with the uptake of cytochrome c from the medium, and the viable count after cytochrome treatment in 0.5 mM acetic acid was reduced as compared with treatment in a water suspension of the cells. The action of cytochrome c on the cells in slightly acid medium was more uniform and more efficient.

Only small amounts of cytochrome c could be removed from the cells by washing with water or 1.0 M sorbitol. Treatment with electrolytes, however, released much of the pigment into the surrounding medium (Table 1).

The penetration of cytochrome c into yeast cells was examined by UV micrography. For comparison and reference, cells of *C. utilis* from minimal medium (Fig. 2a to c) and from medium containing sulfur amino acids (Fig. 2d to f) are shown; the latter contain S-adenosylmethionine in their vacuoles. The location and relative concentration of the compound are revealed in the micrographs made at 265 nm (Fig. 2f). In the illustrations of cells from minimal medium that had been treated with cytochrome c for 30 min at 30°C in isosmotic mannitol (Fig. 3a to c), a great difference in absorbance at 405 nm (by the cytochrome) in the various cells may be seen (Fig. 3a, d). Comparison of the darkest cells in Fig. 3a with the corresponding images of the same field in Fig. 3b or Fig. 3c revealed that the vacuoles had been damaged. To increase the contrast of the vacuole in the UV micrographs, advantage was taken of the accumulation of the strongly UV-absorbing (260 nm) biological compound S-adenosylmethionine in vacuoles of cells which had been cultivated in medium supplemented with sulfur amino acids (10). S-adenosylmethionine-containing cells that were treated with cytochrome c for 30 min at 30°C in isosmotic mannitol are shown in Fig. 3d to f. At this time, cytochrome c had entered the cytoplasm (Fig. 3d) but had not damaged the vacuole (Fig. 3f). When such cells were transferred from isosmotic conditions to water, the vacuoles were broken with the results shown in Fig. 4a to c. Cells harvested during early phases of growth showed greater variation in their response to cytochrome c than those harvested when growth was complete; buds often remained devoid of cytochrome c, whereas mother cells had taken up the material in copious amounts (Fig. 4d to f). When sufficient cytochrome c was provided and the pH was below 6, the buds were also penetrated.

Further experiments were aimed at testing the binding capacity of the cell wall for exogenous cytochrome c and determining whether the protective action of salts, such as phosphate buffer, against cytochrome c occurs by blocking penetration through the cell wall or through the membrane. For this, spheroplasts of *C. utilis*
were prepared, washed, suspended in 1.0 M sorbitol, and treated with cytochrome c. The quantities of cytochrome c necessary to act on the spheroplasts were similar to those effective with whole cells, as indicated by the release of UV-absorbing material into the medium. Figure 5 shows that 0.05 M potassium phosphate buffer (pH 5.7) protected the spheroplasts, but that they were destroyed by cytochrome c in the absence of the buffer. The presence of cytochrome c in the supernatant fluid may be explained by incomplete adsorption to the spheroplasts and by release into the medium on bursting. After the action of cytochrome c on spheroplasts in isotonic medium, microscopic examination revealed debris only; spheroplast ghosts were not seen.
DISCUSSION

Direct evidence for penetration of the yeast cell wall and membrane by protein has been provided in the present experiments by UV micrography of the intracellular localization of cytochrome c taken up from the medium. Most of the cytochrome c that entered the cells was confined to the cytoplasm. If it had also penetrated the vacuole, the micrographs made at 405 nm would probably have appeared uniformly dark and it would have been difficult to determine whether the cytochrome was limited to a uniform surface coating or was uniformly distributed throughout the cell. The enrichment
Fig. 4. UV micrographs of yeast cells treated with cytochrome c. The cells of Fig. 4a, b, and c correspond to those of Fig. 3d, e, and f, except that vacuolar contents were lost because the cells had been exposed to osmotic shock by transfer from 1.0 M mannitol into water. Cytoplasmic shrinking may be noted. Micrographs d (405 nm), e (297 nm), and f (265 nm) show variations in response of S. cerevisiae cells and buds to cytochrome c. The dark appearance of some cells in f is caused by the combined absorbance of cytoplasmic compounds and the large quantity of cytochrome c present (see d). At 265 nm, the absorbance of cytochrome c is 20% of that at 405 nm.

of the vacuole with S-adenosylmethionine by special culture conditions previous to cytochrome treatment made it easy to determine the condition of the vacuole by UV micrography.

When yeast cells were exposed to cytochrome c there were usually some survivors (14). UV micrography revealed this to be due to the heterogeneity of the response to cytochrome c; some cells were not affected and buds in particular were more resistant than mature cells (Fig. 4d to f).

Our micrographic records show that the cell wall does not constitute an effective barrier to cytochrome c. The molecular weight of cytochrome c is about 12,000 and it has a compact conformation (2.5 by 2.5 by 3.7 nm). The di-
Fig. 5. Effect of cytochrome c on spheroplasts of C. utilis. The spheroplasts were exposed for 30 min at 30°C in 1.0 mM sorbitol containing 0.5 mM acetic acid. The quantities of cytochrome c employed are indicated on the abscissa. The samples were cleared by centrifugation, and the supernatant fluid was examined by spectrophotometry at 260 nm for the presence of cytoplasmic constituents and at 408 nm for cytochrome c. The values at 260 nm are corrected for spontaneous bursts of spheroplasts and for the absorbance of cytochrome c.

For reference, the extraction of the spheroplasts was carried out with 1.5 mM perchloric acid; after centrifugation, the supernatant fluid showed a value of 2.1 absorbance units per ml. Symbols: (■), cytoplasmic components released into the medium, measured at 260 nm; (○), same experiment in the presence of 0.05 mM phosphate buffer, pH 5.7; (△), cytochrome c in the supernatant fluid, measured at 408 nm; (△), same experiment in presence of 0.05 mM buffer.

dimensions of this protein are such that it is necessary to revise the current concepts of the size of pores in the yeast cell wall.

The effect of cytochrome c on the membrane has not yet been explained. A plausible hypothesis is that it causes distortions of the membrane by multiple ionic attachment to the acid groups. This may engender lesions that permit more protein molecules to penetrate into the cytoplasm.

The lesions in the membrane are permanent, as evidenced by the possibility of removing much of the cytochrome c by salt or buffer extraction under conditions which do not remove constituents of intact cells (Table 1). This indicates some mode of adsorption or binding of cytochrome c to intracellular components from which it is displaced by salts.

The permanence of the membrane damage by cytochrome c argues strongly against pinocytosis (4) as an explanation of the mechanism of penetration into the yeast cell. Pinocytosis is not usually attended by cell death, or by the loss of most cytoplasmic constituents of low molecular weight, as was seen in the present experiments.

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LITERATURE CITED