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Inhibition of transport systems in yeast by photodynamic treatment with Toluidine blue

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Photodynamic treatment of yeast with the sensitizer Toluidine blue results in loss of cell viability. In previous investigations it was suggested that plasma membrane damage might be responsible for the loss of colony forming capacity. In this context the influence of photodynamic treatment on transmembrane transport systems was studied. It appeared that the uptake of the sugars glucose, lactose and galactose, the amino acids arginine, phenylalanine, glycine and aspartic acid and of the inorganic compound phosphate was inhibited by photodynamic treatment. The different elements of the energy providing system necessary for active transport, viz. the plasma membrane ATPase and the protonmotive force, were not significantly affected by Toluidine blue and light, indicating that inhibition of transport is not caused by a reduction of the membrane potential or the transmembrane pH gradient. These observations suggest that the transport carriers themselves were damaged by treatment with Toluidine blue and light. This could be confirmed in experiments, in which the lactose and galactose transport proteins of treated and untreated cells were reconstituted in plasma membrane vesicles. It appeared that the carriers, obtained from photodynamically treated *Kluyveromyces marxianus* cells, had lost their transport capacity.

Introduction

Photodynamic treatment of yeast with the singlet oxygen producing sensitizer Toluidine blue (TB) causes loss of colony formation [1]. TB is a positively charged dye that under physiological conditions does not, or very slowly, enter the yeast cell [1]. It interacts, however, with negatively charged groups on the cell surface, such as periplasmatically localized polyphosphates [2]. The mechanism of the inactivation of the yeast is not fully understood. Data in the literature seem to indicate that genetic damage is not the primary cause of loss of clonogenicity [1]. An obvious target for the photodynamic attack by TB is the plasma membrane. Since the plasma membrane controls the access of nutrients and prevents the leakage of various essential compounds, damage to the membrane could be involved in cell killing. It has indeed been shown that the plasma membrane is damaged by TB and light, but the damage reported, i.e., an all-or-none disruption of the barrier properties, was not causally related to loss of clonogenicity [3]. In the same paper it was suggested that instead of a gross disruption of the membrane, a more subtle modification of the plasma membrane properties could be involved in photodynamically induced loss of clonogenicity. In this context the effect of photodynamic treatment on transport was studied. In this paper it will be shown that sugar, amino acid and phosphate transport across the plasma membrane is inactivated by photodynamic treatment with TB. Furthermore, evidence will be presented, indicating that transport inhibition is not caused by a collapse of the energized state needed for active transport, but rather by photodynamic damage of the transport proteins themselves.

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Abbreviations: TB, Toluidine blue; pmf, protonmotive force; $\Delta\psi$, transmembrane potential; ΔpH, transmembrane proton gradient; TPP, tetraphenylphosphonium.

Materials and Methods

Toluidine blue O was obtained from Serva, nitrocellulose filters (pore size 0.45 μm; BA 85) from Schleicher and Schuell, Scintillator 299 from Packard and *Escherichia coli* phospholipids P6398 from Sigma.

D-[^{14}C]Glucose (0.1 GBq/mmol), L-[^{14}C]glucose (2.04 GBq/mmol), [^{14}C]lactose (2 GBq/mmol), [^{14}C]maltose (20 GBq/mmol), [^{14}C]galactose (1.9 GBq/mmol), [^{14}C]arginine (11.3 GBq/mmol), [^{14}C]aspartic acid (8.4 GBq/mmol), [^{14}C]phenylalanine (19.4 GBq/mmol), [^{14}C]glycine (4.1 GBq/mmol), [^{32}P]orthophosphate and [^{14}C]tetraphenylphosphonium (1.2 GBq/mmol) were obtained from Amersham, and [^{14}C]methylamine (2.2 GBq/mmol) from NEN.

Yeast cells of the strain *Kluyveromyces marxianus* CBS 397 were grown aerobically at 30°C in a complex medium containing 1% bacto-peptone, 1% yeast extract and 2% glucose. The doubling time or generation time of this yeast was 90 min. The culture was grown to the early stationary phase (19 h of incubation), having a cell density of approx. $4 \cdot 10^8$ cells/ml. Subsequently, the cells were harvested and washed twice with deionized water. For photodynamic treatment the cells were resuspended in 1 mM Tris-HCl (pH 7.2) at a cell density of 0.5% wet (w/v). A 10 ml aliquot of the cell suspension was transferred to a 25 ml Erlenmeyer flask. After 20 min of incubation in the dark with 1 μg/ml Toluidine blue the cells were illuminated with a 150 W halogen lamp under continuous stirring. A Kodak wratten No. 8 filter was used, cutting off at wavelengths < 500 nm. An infrared filter prevented heating of the suspension. The temperature never exceeded room temperature. The fluency was about 300 W/m^2 as measured by an EG&G Electro-optics model 450–1 photometer.

For transport studies 0.5 or 1 ml samples of the cell suspension were taken, centrifuged and the pellet was resuspended with 125 μl 50 mM Tris-maleate (pH 4.9) containing 0.5% ethanol and 100 μl suspension was transferred to a vessel at 30°C. After 1 min of preincubation under aerobic conditions, 10 μl of radioactively labeled substrate was added. The uptake was stopped after an appropriate incubation time by adding 2 ml ice-cold 0.1 M LiCl and the suspension was filtrated through a nitrocellulose filter followed by one wash with 2 ml ice-cold LiCl. The filter was added to Scintillator 299 and radioactivity was measured. Measurement of the [^{14}C]tetraphenylphosphonium uptake was performed according to Van den Broek et al. [4]. Initial transport velocities were measured over a period of 10 s for glucose, galactose, arginine, phosphate, 30 s for lactose, aspartic acid, phenylalanine, glycine and 3 min for methylamine and TPP. Uptake was linear with time over these periods. Uptake velocities were calculated after correction for binding to filters and cells.

Intracellular pH was determined using the method of Borst-Pauwels and Dobbelmann [5]. After photodynamic treatment, the cells were washed and resuspended in water at a cell density of 0.5% (w/v). H^+ uptake was initiated by addition of HCl to obtain a medium pH of 4. Proton influx was measured with a conventional pH electrode connected to a pH meter, equipped with a recorder.

Plasma membranes were isolated as described before [6]. Membrane vesicles were prepared by fusion of plasma membranes with proteoliposomes containing cytochrome-*c* oxidase as a protonmotive force generating system according to the technique described by Van Leeuwen et al. [6]. *E. coli* phospholipids were used as the source of liposomal lipids. Vesicles were prepared at pH 5.7 to obtain optimal transport velocities, as described before by Van Leeuwen et al. [7]. Sugar transport and measurements of the protonmotive force in vesicles were carried out according to Van Leeuwen et al. [6].

Plasma membrane ATPase activity in purified membranes was measured in a buffer containing 25 mM Tris-maleate, 50 mM KCl and 5 mM $MgCl_2$ at pH 6.0. ATP was added to a final concentration of 4 mM. After 60 min at 30°C the amount of orthophosphate liberated was determined as described by Tijssen et al. [8].

Cell survival was determined by taking 10-μl samples, diluting these samples to 5000 cells/ml and spreading 500 cells on solidified (1% agar) medium. The number of colonies was counted after incubation for two days in the dark at 30°C.

The data shown are representative of a typical experiment carried out at least three times in which similar results were obtained.

Results

Photodynamic effects on transport velocity

The influence of photodynamic treatment with TB on transport activity was studied by measuring the uptake of a wide variety of substrates. First it was established that TB alone did not have an effect on transport activity or clonogenicity. As shown in Fig. 1, uptake of lactose, glucose and galactose was already inhibited after relatively short illumination periods in the presence of TB. These so called 'high affinity' transports proceed through proton/sugar symport mechanisms in which the electrochemical proton gradient across the membrane, provides the energy required for solute uptake against its concentration gradient. In *K. marxianus*, glucose can also be transported by an other separate transport system working with low affinity [9]. This transport is measurable at an external concentration of 5 mM and, as shown in Fig. 1, it was also strongly inhibited by treatment with TB and light. Photodynamic treatment does not only inhibit carbohydrate uptake but it also affects transport of various other compounds as shown in Fig. 2. From the four amino acids studied the transport of the positively charged arginine turned out to be the most sensitive towards photodynamic treatment. Fig. 2 indicates that

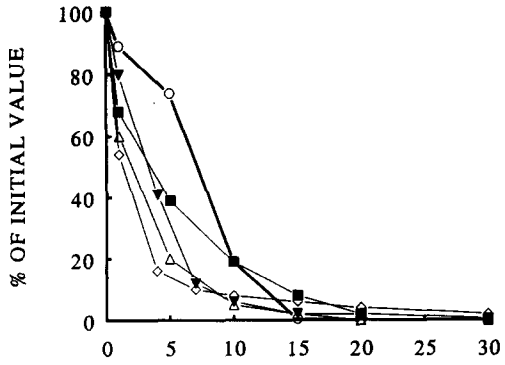

Fig. 1. The influence of photodynamic treatment on sugar transport and colony formation. Initial concentration of substrates and initial transport velocities (100% values) were: glucose 60 μM, 0.3 nmol/min per mg yeast (△); lactose 6 μM, 16 pmol/min per mg yeast (◇); galactose 10 μM, 0.1 nmol/min per mg yeast (▼) and glucose 5 mM, 8.7 nmol/min per mg yeast (■). Colony formation (○). Transport velocities of controls (cells without TB+light and cells+TB−light) were similar to the presented initial transport values.

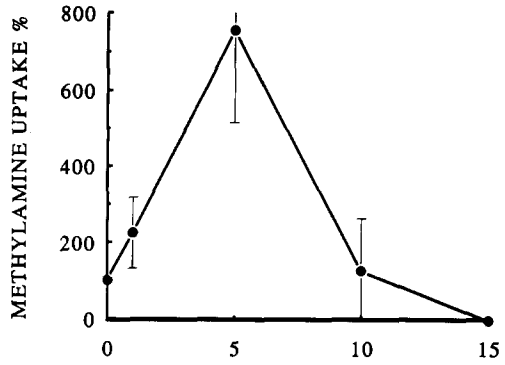

Fig. 3. Methylamine uptake during photodynamic treatment. Initial methylamine concentration 7 μM. Initial transport velocity 0.17 pmol/min per mg yeast. Error bars indicate the standard deviation, $n = 6$. Transport velocities of controls (cells without TB+light and cells+TB−light) were similar to the presented initial transport values.

also the uptake of phosphate was inhibited during treatment. In contrast to sugar, amino acid and phosphate transport, the uptake of methylamine, proceeding through the ammonia transporter (Ref. 10 and references therein), was stimulated at the initial phase of illumination. After 15 min of treatment transport was completely inhibited (Fig. 3).

Photodynamic effects on the protonmotive force

Figs. 1–3 indicate that all transport systems studied, except for methylamine uptake, were inhibited at a rate equal to or even faster than the loss of clonogenicity. It seemed feasible that the inhibition of all these different transport systems would be caused by one common event, viz. the photodynamically induced collapse of the driving protonmotive force. The main generator of the pmf in yeast is the membrane-bound H^+-ATPase, which was not very sensitive to this photodynamic treatment (Table I). Measurements of the plasma membrane ATPase activity at pH 6 showed only a slight decrease in activity during early photodynamic treatment. After 10 min of illumination more than 80% of the ATPase activity was still present, only after prolonged light exposure the ATPase became inactivated.

Proton influx measurements showed that photodynamically treated yeast cells did not have an increased proton permeability up to 10 min of treatment (data not shown). Therefore, cells should still be able to maintain a proton gradient.

The internal pH was determined to be 6.65 in control cells and 6.60 or 6.35 in cells illuminated for 5,

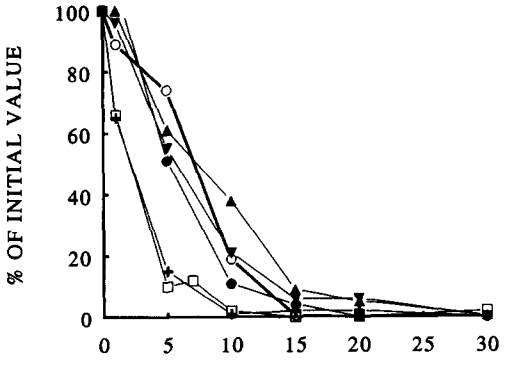

Fig. 2. The influence of photodynamic treatment on colony formation, uptake of amino acids and phosphate. Initial concentration of substrates and initial transport velocities (100% values) were: arginine 1 μM, 15 pmol/min per mg yeast (+); phenylalanine 0.8 μM, 4 pmol/min per mg yeast (▲); aspartic acid 2 μM, 1.3 pmol/min per mg yeast (●); glycine 10 μM, 7.5 pmol/min per mg yeast (▼) and orthophosphate 0.1 mM, 2.4 nmol/min per mg yeast (□). Colony formation (○). Transport velocities of controls (cells without TB+light and cells+TB−light) were similar to the presented initial transport values.

TABLE I

Effect of photodynamic treatment on plasma membrane ATPase activity

ATPase activity was measured as described in Materials and Methods. The observed activities could be inhibited for more than 80% by 100 μM vanadate. Less than 3% inhibition was achieved in the presence of 10 mM sodium azide. The enzyme activity taken as 100% was 1.1 μmol orthophosphate liberated/min per mg protein.

Illumination time (min)	ATPase activity (%)
0	100
5	95
10	83
25	8

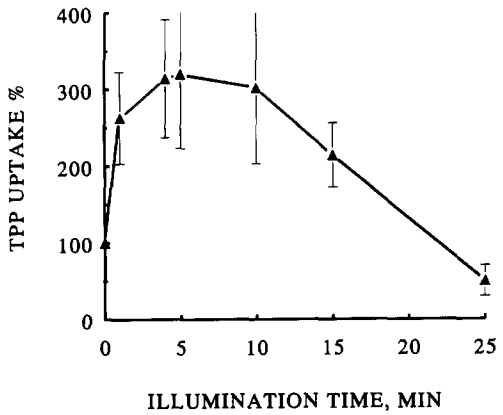

Fig. 4. Tetraphenylphosphonium uptake during photodynamic treatment. Initial TPP concentration 1 μM. Initial transport velocity 0.12 pmol/min per mg yeast. Error bars indicate the standard deviation, $n = 8$. Transport velocities of controls (cells without TB + light and cells + TB − light) were similar to the presented initial transport values.

respectively, 10 min. Only after prolonged illumination the intracellular pH dropped to levels under pH 6. These results indicate that initially ΔpH is not significantly affected by photodynamic treatment. Finally, the $\Delta\psi$ was probed by measurements of tetraphenylphosphonium influx [11]. Since TPP uptake is extremely slow in this yeast, an accurate determination of $\Delta\psi$ can not be made [11]. However, influx of this membrane potential probe gives a reliable indication of changes in $\Delta\psi$ [11]. The TPP uptake increased in the first 10 minutes of illumination and only decreased after prolonged light exposure (Fig. 4). This indicates that $\Delta\psi$ increased at illumination times where transport was already inhibited. Taken together, these data demonstrate that the pmf was not decreased by photodynamic treatment at illumination times where transport systems were already inactivated.

Photodynamic effects on the carrier activity

Pmf-driven sugar transport in *K. marxianus* can well be measured in membrane vesicles containing the pmf generator cytochrome-*c* oxidase [6]. In this system, carrier-mediated transport of galactose and lactose can be distinguished from passive diffusion by comparing its transport with the uptake of L-glucose and maltose, respectively. Since this yeast strain does not contain specific transporters for the latter two sugars, uptake of L-glucose and maltose in vesicles can only proceed by passive diffusion. In the absence of a pmf, galactose and lactose uptake proceed by passive diffusion and facilitated transport through the proton symport carrier [6,7]. Fig. 5 shows that upon the subsequent energization, by addition of ascorbate, tetramethyl-*p*-phenylenediamine and cytochrome *c* ($t = 6$ min), a clear stimulation of the galactose and lactose uptake was measured. Galactose uptake velocities, measured 1 min after initiating transport, were 0.64, 0.14 and 0 nmol sugar/min per mg protein in vesicles prepared from cells which were photodynamically treated for, respectively, 0, 5 and 10 min. This shows that in vesicles prepared from cells that were exposed for 5 min to TB and light about 22% of the original transport activity was present. 10 min of treatment was sufficient to reduce the activity to the passive diffusion level. The pmf, measured as described before [6] had values similar to the ones descibed by Van Leeuwen et al. [6]. Moreover, in all samples the pmf generated by cytochrome-*c* oxidase was identical and could therefore not explain the inhibition of transport (data not shown).

Repair of photodynamically induced transport inhibition

The possible repair of transport functions was studied in post-incubation experiments. Yeast cells were treated with TB and light to a level at which 40% of the cells were still able to form colonies, coinciding

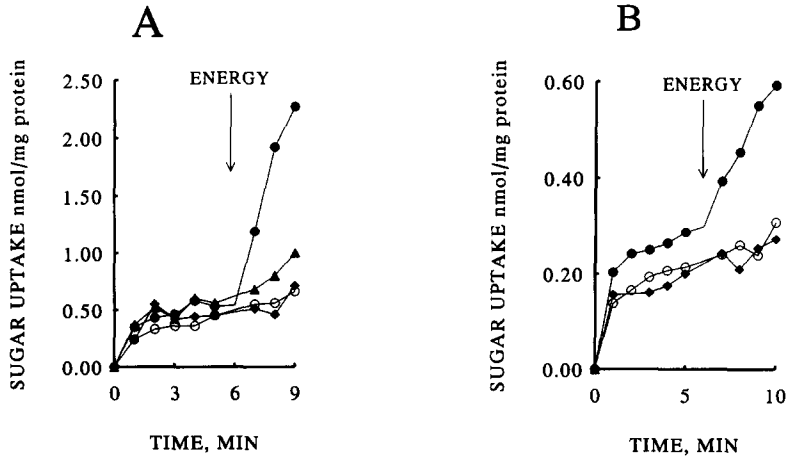

Fig. 5. Sugar uptake in membrane vesicles prepared from photodynamically treated yeast. (A) D-Galactose transport. Initial sugar concentration: 60 μM. Galactose uptake, cells not illuminated (●), cells 5 min illuminated (▲) and 10 min illuminated (◆); L-glucose transport (○). (B) Lactose transport. Initial sugar concentration: 30 μM. Lactose uptake, cells not illuminated (●), cells 10 min illuminated (◆). Maltose transport (○).

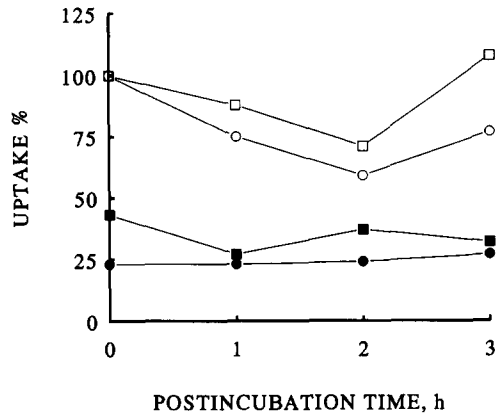

Fig. 6. Postincubation in complex medium. Cells were illuminated until 40% of viability was reached, followed by a post incubation at room temperature. Uptake was corrected for slight variations in cell number. Glucose uptake (initial concentration 60 μM) cells not illuminated (○), glucose uptake cells illuminated (●), phosphate uptake (initial concentration 0.1 mM) cells not illuminated (□), phosphate uptake cells illuminated (■). Initial transport velocities were identical to the ones presented in Figs. 1 and 2.

with glucose and orthophosphate uptake rates that were reduced to 25% and 45%, respectively. After photodynamic treatment the cells were transferred to complex medium and the uptake of glucose and orthophosphate was measured 1, 2 and 3 h after treatment. During this period no significant change in cell number was measured. As shown in Fig. 6 neither glucose transport nor phosphate transport were restored in this period.

Discussion

Photodynamic treatment of yeast cells results in loss of clonogenicity. Previous publications had suggested that this could be caused by damage at the level of the plasma membrane [1,3]. This paper shows that transport of various sugars, amino acids and of phosphate was inhibited in *K. marxianus* by treatment with TB and light. Many solute transport systems of this yeast proceed by a pmf-dependent mechanism. This has been shown, e.g., for the lactose specific transporter [12] as well as for the carrier that catalyzes the high-affinity uptake of both galactose and glucose [6,13]. For those pmf-dependent transports, the photodynamically induced inhibition can be caused in two ways: either the absence of a proton gradient across the plasma membrane or a damaged transport protein. The experimental results rule out the former possibility. First, the plasma membrane ATPase, being the main generator of the proton gradient, was not substantially affected by this treatment. Also, no indication was found that the plasma membrane became leaky for protons after illumination up to 10 min. The integrity of the plasma membrane was confirmed by the observation that in this period the pmf was not dissipated. Most likely the pmf remains rather constant during the first 10 min of light exposure, since the slight decrease in the internal pH, and thus ΔpH, was compensated by an increase in $\Delta\psi$. Variation in pmf can therefore not explain the observed inhibition of transport. The second option, a damaged carrier, was investigated by reconstituting the sugar-proton symporters into vesicles. The use of this in vitro system has the advantage that transport can be measured without any influence of metabolism, and independent of the plasma membrane ATPase. Measurements in this in vitro system thus provide direct information about the carrier activity. Since inhibition of galactose and lactose transport in vesicles was observed, the conclusion must be that treatment of yeast with TB and light results in inactivation of both the lactose and the galactose specific proton symport carriers.

The fact that both pmf-driven sugar transporters were damaged, connected with the fact that the pmf in whole cells was not negatively influenced by photodynamic treatment strongly indicates that energy driven solute uptake carriers are directly attacked by TB and light. This might hold also for the amino acid and phosphate uptake systems measured in this study. The exact mechanism of amino acid and phosphate transport in *K. marxianus* is not yet known, but it can be assumed that, analogous to the situation in *Saccharomyces* and *Candida*, uptake of most of these compounds is coupled to the pmf or its components [14,15]. Unfortunately these transporters have a relatively low abundance and can therefore not be monitored accurately in the reconstituted vesicle system.

An interesting observation is the fact that almost all transport systems measured were susceptible to photodynamic treatment. Only methylamine transport and the proton-translocating plasma membrane ATPase were initially resistant to this treatment. They became inactivated at times where the plasma membrane had already lost its barrier properties. The rate of inactivation of sugar, amino acid and phosphate transport is to a large extent the same for these chemically different substances. An explanation might be that all these different transport systems contain a comparable sensitive site, except for the methylamine carrier that would not posses such a freely accessible site. For a number of protonmotive force-driven transport systems in yeast, it has been suggested that a histidine residue would be essential for the carrier activity [7,16,17]. This could be a general phenomenon for pmf-driven transport, since histidine residues are also essential for cotransport activity in bacteria [18]. The photodynamic inactivation of the transport systems could occur through modification of this essential histidine residue, as histidine is one of the most sensitive amino acids towards photodynamic treatment [19,20].

In a previous paper [3] it was suggested that a subtle modification of functions, associated with the plasma membrane, could be responsible for the photodynamically caused loss of clonogenicity. Indeed, the results presented in this study show that photodynamic treatment with the sensitizer Toluidine blue causes modification of plasma membrane functions, by damaging various membrane proteins. In general, the inactivation of one transport system alone will not result in the observed loss of colony forming capacity of this wild-type yeast. The massive reduction of transport capacity of a wide variety of solutes, as observed in this paper, will lead to shortage of essential substrates for anabolic and catabolic processes and will therefore certainly contribute to the loss of clonogenicity. Whether this can fully explain the observed loss of clonogenicity or that other factors are also involved, is a subject for further research.

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