Possible nystatin-protein interaction in yeast plasma membrane vesicles in the presence of ergosterol. A Förster energy transfer study

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Abstract The Förster energy transfer from tryptophan residues of membrane proteins to nystatin was measured in reconstituted yeast plasma membrane vesicles free of, or doped with, ergosterol. We wanted to elucidate whether the functional change of membrane transport proteins from H^+ symporters to facilitators, observed in ergosterol-containing plasma membrane vesicles on addition of nystatin [Opekarová and Tanner (1994) FEBS Lett. 350, 46–50], is reflected in altered protein-nystatin relations within the membrane. Both frequency-domain and time-domain time-resolved fluorescence spectroscopy showed that in the presence of ergosterol nystatin is located much closer to membrane proteins than in its absence.

Key words: Nystatin; Yeast plasma membrane vesicle; Arginine transport; Förster energy transfer; Tryptophan fluorescence; Fluorescence lifetime

1. Introduction

Like other polyene antibiotics, nystatin is claimed to interact specifically with ergosterol in the yeast plasma membrane; the consequence of this interaction is pore formation or membrane fluidity changes. As a result, intracellular metabolites can freely leave the interior of the cell, which eventually leads to cell death (for review see [1,2]).

Treatment with nystatin has been reported to bring about a rapid release of previously accumulated amino acids such as arginine and histidine [3-5] and other metabolites such as allantoate [6], urea [7], allantoin [8], oxalurate [9] and γ-aminobutyrate [10] from the yeast. These substances are released from the cells irrespective of their cellular distribution. Urea is located mainly in the cytoplasm, while basic amino acids, allantoin and allantoate are predominantly sequestered in vacuoles.

Recently we examined the effect of nystatin on the transport of arginine in an in vitro system of yeast plasma membrane vesicles which eliminates the interference of cellular compartmentation [11] and offers the possibility to change nearly arbitrarily the membrane lipid composition. Arginine accumulation in the vesicles is driven by protonmotive force (pmf); however, even when the pmf is completely abolished by low concentrations of uncouplers no efflux of accumulated arginine occurs [11]. This finding reflects the unidirectionality of amino acid transport observed in whole yeast cells (for review see, e.g. [12]). Addition of low concentrations of nystatin (5–10 μg/mg lipid) to the vesicles without ergosterol did not affect the arginine fluxes whereas in ergosterol-containing vesicles it caused a massive efflux of the accumulated arginine while the magnitude of the protonmotive force in the system remained largely unaffected. These results suggest that nystatin, in addition to its well-established binding to ergosterol, exhibits another effect in the yeast plasma membrane: at low concentrations it obviously alters the properties of the membrane carriers, converting them from H^+ symporters to facilitators.

These transport data pointing to nystatin affecting membrane proteins can be confirmed by physical methods, e.g. by measuring the Förster energy transfer (FRET) [13] which makes it possible to estimate the distance between a donor (i.e. tryptophan in membrane proteins) and an acceptor (nystatin). The average distance was estimated by measuring the lifetime of tryptophan residues in vesicles prepared from yeast plasma membranes fused either with *E. coli* phospholipids (approx. 50% of phosphatidylethanolamine) without ergosterol, or in the same vesicles enriched with 12% (w/w) of ergosterol.

2. Materials and methods

2.1. Materials

L-α-Phosphatidylethanolamine (type IX from *E. coli*, approx. 50%), ergosterol and nystatin were obtained from Sigma.

2.2. Yeast strains

A transformed strain *Saccharomyces cerevisiae* RS 453 (CAN1) (ade 2-1, leu 2-3,112, his 3-11,15, ura 52) with a multicopy plasmid pWHY bearing the CAN1 gene coding for arginine permease [14] was used in the study.

2.3. Media and growth conditions

The yeast strain *S. cerevisiae* RS 453 (CAN 1) was grown on a yeast minimal medium containing 0.67% yeast nitrogen base without amino acids, 2% glucose and necessary bases and amino acids.

2.4. Isolation of plasma membranes

Plasma membranes were isolated from yeast grown at 29°C on a rotary shaker. At $A_{578} = 0.5$–0.8 the cells were harvested, washed and broken by shaking with glass beads (diameter 0.45–0.5 mm). The plasma membranes were isolated essentially according to [15]. During

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Abbreviations: FRET, Förster energy transfer; PL, *E. coli* phospholipid liposomes; PE, ergosterol-containing *E. coli* liposomes; PLM, yeast membranes fused with the phospholipid liposomes; PEM, membranes fused with ergosterol-containing liposomes; χ_R^2 the reduced chi-square; FWHM, full-width of the half-maximum

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the isolation a mixture of 0.25 M 4-aminobenzamidine dihydrochloride and 0.5 M phenylmethylsulphonyl floride was repeatedly added in amounts of 1/100 of the actual isolation solution volume. The isolated plasma membranes, suspended in 50 mM potassium phosphate, pH 6.3, 20% glycerol and 1 mM EDTA, were rapidly frozen in liquid nitrogen and stored at −80°C.

2.5. Preparation of liposomes

E. coli phospholipid liposomes (PL) were prepared as described by Opekarová et al. [14]. For preparation of ergosterol-containing liposomes (PE), chloroform solutions of ergosterol and *E. coli* phospholipid were mixed so that the final concentration (w/w) of ergosterol was 12%. The subsequent procedure was the same as above.

2.6. Fusion of liposomes with yeast plasma membranes

The liposomes were mixed with yeast plasma membranes at a ratio of 20:1 (lipid/protein) in a final volume of 100–200 μl. The resulting plasma membrane vesicles (PLM, membranes fused with phospholipid liposomes; PEM, membranes fused with ergosterol-containing liposomes) were supplemented with 1 mM $MgSO_4$ and rapidly frozen in liquid nitrogen. Before use the fused membranes were slowly thawed at room temperature and briefly sonicated with a probe-type sonifier (Cole-Palmer Instruments Co.).

Measurements with individual preparations of liposomes and vesicles (PL, PE, PLM, PEM) were performed in 50 mM potassium phosphate buffer, pH 6.3, containing 2 mM $MgSO_4$. Nystatin (stock solution 10 mg/ml in DMSO) was added to a final concentration of 5 μg/mg lipid.

2.7. Protein estimation

Protein was determined by the method of Bradford [16].

2.8. Dynamic fluorescence measurements and data analysis

2.8.1. Frequency-domain measurements. Lifetime measurements were performed with a multifrequency phase fluorometer (ISS K2) interfaced with a PC 486 computer for data collection and analysis. The excitation wavelength was 280 nm (xenon arc lamp), emission being observed at 350 ± 10 nm. Ten modulation frequencies between 5 and 200 MHz were used. All measurements were obtained using 1,4-bis[2-5(phenyl)oxazolyl]benzene (POPOP, lifetime 1.35 ns; see [13]) in the reference cell. The temperature of the samples was maintained at 25°C with an external bath circulator. Measurements and data analysis were performed according to [20]. The optical density of the sample was maintained at A_{360}=0.1. Corrections for background fluorescence and/or light scattering were made by performing the frequency-domain measurements with phospholipids without proteins as control sample and then correcting the data as in [17]. Notably, the addition of nystatin in DMSO resulted in an appearance of a much higher background signal in the control samples (liposomes without proteins PL and PE), which required the above correction.

The experimental data were analyzed assuming either a sum of exponentials or a continuous distribution of lifetime values. A program provided by ISS (La Spezia, Italy) was used for the distribution analysis. For both exponential and distribution analysis, this program minimizes the reduced chi-square, χ_R^2 [18]. χ_R^2 was used to judge the goodness of fit of calculated functions with phase and modulation data.

2.8.2. Time-domain measurements. The method of time-correlated single-photon counting was employed as reported elsewhere [19] with instrumentation and data analysis as described previously [21]. The excitation wavelength was 280 nm and emission was observed at 350 ± 10 nm.

2.9. Förster energy transfer (FRET) calculations

Calculated distances between donor and acceptor pairs can be derived from Förster energy transfer measurements. The apparent efficiency E of energy transfer between a donor and an acceptor is dependent on the decrease in the donor lifetime after addition of an acceptor as folows:

$$E = 1-(\tau_{DA}/\tau_A) \quad (1)$$

where τ_{DA} and τ_A are the lifetimes of the donor in the presence and absence of the acceptor, respectively [13]. The efficiency E is related to the absolute rate of energy transfer k_{ET} as given below:

$$k_{ET} = \frac{1}{\tau_{DA}} - \frac{1}{\tau_D} \quad (2)$$

where τ_D and τ_{DA} are the donor lifetimes in the absence and in the presence of the acceptor, respectively. If the donor has a continuous lifetime distribution rather than discrete lifetime(s), τ_D and τ_{DA} should be replaced by mean values calculated over the distributions.

For a two-component Lorentzian distribution, the mean reciprocal lifetime $1/\tau_R$ can be expressed as

$$\frac{1}{\tau_R} = \frac{\alpha_1 \cdot w_1}{\alpha_1 \cdot w_1 + \alpha_2 \cdot w_2} \cdot \frac{\tau_1}{\tau_1^2 + w_1^2} + \frac{\alpha_2 \cdot w_2}{\alpha_1 \cdot w_1 + \alpha_2 \cdot w_2} \cdot \frac{\tau_2}{\tau_2^2 + w_2^2} \quad (3)$$

where α_1, α_2, τ_1, τ_2, w_1, w_2 are the fractions, centres and full widths at half-maximum (FWHM) of the first and second component, respectively, $\alpha_1+\alpha_2=1$, and the fractions refer to non-normalized Lorentzians, i.e. they are peak heights rather than areas under the peak.

Alternatively, we used the median as another, more standard mean value to calculate the FRET rates. The median τ_M is the value that divides the area under the distribution function into equal parts; for a two-component Lorentzian lifetime distribution, it can easily be found as the solution of the following equation:

$$\alpha_1 \cdot w_1 \cdot \mathrm{arctg}\left(\frac{\tau_M - \tau_1}{w_1}\right) + \alpha_2 \cdot w_2 \cdot \mathrm{arctg}\left(\frac{\tau_M - \tau_2}{w_2}\right) = 0 \quad (4)$$

3. Results and discussion

In order to prove an altered relationship between membrane proteins and nystatin in the presence of ergosterol, the FRET technique was used to estimate the distance between tryptophan residues in the proteins and nystatin in

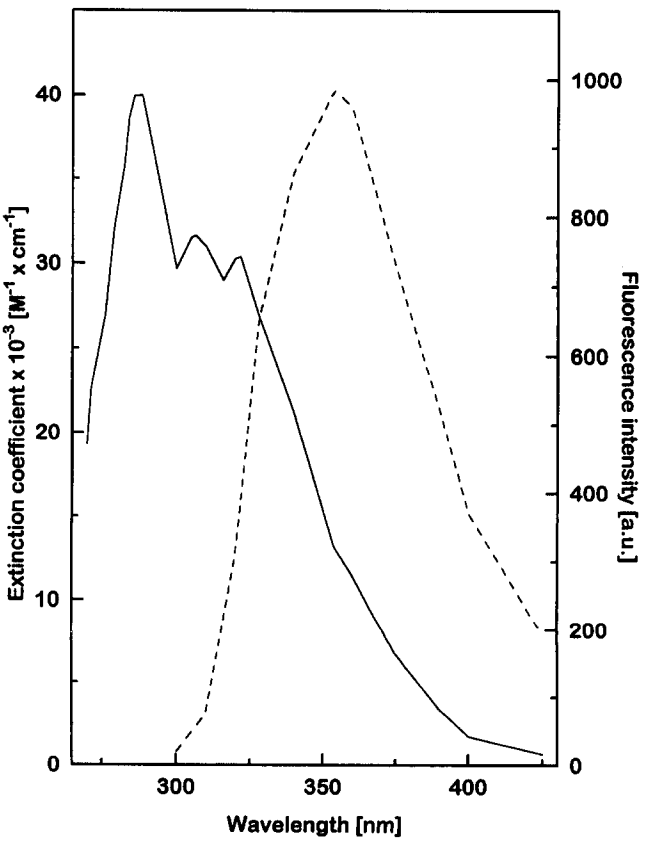

Fig. 1. Extinction coefficient of nystatin (solid line) and fluorescence emission spectrum of tryptophan (dashed line) in 50 mM potassium phosphate buffer, pH 6.3. The excitation wavelength was 280 nm. The calculated critical distance R_0=2.8 nm.

Table 1
Two-component lifetime distribution analysis of tryptophan

Sample	τ_1 (ns)	w_1 (ns)	τ_2 (ns)	w_2 (ns)	f_1/f_2	τ_M (ns)	τ_R (ns)	χ_R^2
PLM	3.6	4.1	0.10	0.05	49.0	3.5	4.2	4.2
PEM	3.4	3.7	0.10	0.05	49.0	3.4	4.2	4.6
PLM+N	3.9	0.3	0.10	0.05	4.0	3.7	1.0	4.5
PEM+N	3.9	0.1	0.10	0.05	0.5	0.1	0.17	4.8

Fluorescence lifetimes of PLM, PEM (266 μl) in 2 ml potassium phosphate buffer, pH 6.3, without nystatin (PLM, PEM) and with nystatin (5 μl, PLM+N, PEM+N); τ_1, τ_2 represent the calculated lifetime components, w_1, w_2 are the full-widths at half-maximum of the Lorentzian lifetime distributions, and f_1/f_2 is the ratio of fractional intensities of lifetime components ($f_1+f_2=1$). The average lifetimes τ_M and τ_R are the median and the mean reciprocal lifetime, respectively. They were calculated as described in section 2. Reduced chi-square, χ_R^2, denotes the goodness of the fit of calculated functions with measured data.

yeast plasma membrane vesicles. The emission spectrum of tryptophan overlaps well with the absorption spectrum of nystatin (Fig. 1). Thus, tryptophan can serve as an energy donor and nystatin as an acceptor for Förster energy transfer experiments. The calculated critical distance between tryptophan and nystatin in a buffer was R_0=2.8 nm. Hence, in this system it is possible to detect whether or not nystatin comes into close proximity (i.e. up to approx. 6 nm) of the protein, and how the presence of ergosterol affects this distance. To avoid the trivial errors often present in FRET calculations from steady-state spectra, dynamic measurements were performed and the efficiency of Förster energy transfer was calculated from the lifetime of the tryptophan residue.

First, the frequency-domain method was employed; the frequency response of the tryptophan fluorescence was recorded with modulations varying from 5 to 250 MHz and analyzed as described in Section 2. The goodness of fit was evaluated by the reduced chi-square, χ_R^2. The two-component Lorentzian distribution appeared as the best fit for the data, with χ_R^2=4.2 (Table 1). Notably, while the χ_R^2 of the two-component Gaussian distribution was only slightly higher (5.3), the χ_R^2 of the two- or even three-exponential decays was significantly higher (24.3 and 19.5, respectively, data not shown).

The short-lived component of the distribution had the center at τ_2 =0.1 ns and its fraction in the steady-state intensity was less than 2% while the long-lived component was centered at τ_1 =3.6 ns for PLM (pure phospholipid vesicles) or τ_1 =3.4 ns for PEM (ergosterol containing vesicles). The calculated average lifetimes τ_R (mean reciprocal lifetime) were therefore 3.6 and 3.4 ns for PLM and PEM, respectively. The full width at half-maximum (FWHM) of the long-lived components of the Lorentzian distribution was broad (w_1=4.1 for PLM and w_2=3.7 for PEM). This clearly indicated a heterogeneous population of tryptophan residues reflecting a large variety of different membrane proteins in the plasma membrane vesicles. The heterogeneous microenvironment of individual tryptophan residues in individual proteins probably contributes to the heterogeneous response.

The ratio of the long-lived to the short-lived component (f_1/f_2) significantly decreased in the presence of nystatin (see Table 1). The fraction of the long-lived component in steady-state fluorescence intensity decreased from 98% in the absence of nystatin to 80% in its presence in PLM and to 34% in PEM. The average lifetime τ_M (median) was 3.7 ns in PLM, decreasing significantly (to 0.1 ns) in PEM.

Table 2
Characteristic lifetimes of energy transfer

Sample	τ_a (ns)	τ_b (ns)
PEM	1.3 ± 0.10	0.4 ± 0.1
PLM	0.18 ± 0.07	−0.03 ± 0.1

The characteristic lifetimes of energy transfer were obtained by the phase-domain method (τ_a, mean reciprocal lifetime from a two-component Lorentzian distribution) and the time-domain method (τ_b, average lifetime from a double-exponential decay).

An alternative method used to describe the Förster energy transfer was based on the reciprocal value of the mean reciprocal lifetime τ_R (see Table 1). This value decreased from 4.2 in the absence of nystatin to 1.0 and 0.17 in its presence in PLM and PEM, respectively. This clearly indicates the presence of Förster energy transfer in PEM with nystatin. While Förster energy transfer in PLM with nystatin was not clearly demonstrated by the method using τ_M, the method making use of τ_R revealed a partial energy transfer also in this system. Nevertheless, with both methods the energy transfer in PEM was significantly higher than in PLM.

Interestingly, the lifetime distribution was much more homogeneous in the presence of nystatin. This could indicate that one class (or a homogeneous population) of tryptophan residues could remain unaffected by nystatin.

The experiments were repeated three times and the average characteristic lifetimes (i.e. the differences in average lifetimes τ_R in the systems without and with nystatin) of Förster energy transfer were calculated for both systems (PEM and PLM). While the characteristic lifetime of FRET in PEM was 1.3 ± 0.3 ns, in PLM it decreased to 0.18 ± 0.09 ns (see Table 2). These experiments clearly indicated that the energy transfer was more pronounced in PEM than in PLM. In other words, nystatin was in a closer proximity to tryptophan residues (and consequently to the protein) in ergosterol-containing vesicles.

To verify the above results and conclusions obtained with the frequency-domain approach, an independent set of experiments with the same PEM and PLM samples was performed with a different instrumentation using a time-domain method yielding again a Förster energy transfer estimation. As indicated in Table 2, a significantly higher rate of energy transfer was obtained in PEM (characteristic lifetime about 0.4 ns) relative to that in PLM (characteristic lifetime virtually zero). This again indicates a closer proximity of nystatin to proteins in ergosterol-containing vesicles.

The above results, which document that nystatin is located in a much closer vicinity of membrane proteins in the presence of ergosterol than in its absence, indicate a possible interaction of this antibiotic with the proteins. Our data do not permit us to decide whether the effect reflects a direct nystatin-protein interaction or whether it represents merely an ergosterol-mediated change in the relative orientation of nystatin and the proteins. In any case, its result is obviously a change in the functional state of the transport proteins, as seen in physiological studies which demonstrated that nystatin caused changes in the properties of transport systems reconstituted in ergosterol-containing vesicles. Nystatin binding to ergosterol in the vicinity of an amino acid permease released this yeast transporter from its coupling to energy and converted it to a facilitator [11]. A more defined system (e.g. a

single carrier protein reconstituted in the liposomes) is now required to provide further information.

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