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The role of ABC proteins Aus1p and Pdr11p in the uptake of external sterols in yeast: Dehydroergosterol fluorescence study

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ABSTRACT

Uptake of external sterols in the yeast *Saccharomyces cerevisiae* is a multistep process limited to anaerobiosis or heme deficiency. It includes crossing the cell wall, insertion of sterol molecules into plasma membrane and their internalization and integration into intracellular membranes. We applied the fluorescent ergosterol analog dehydroergosterol (DHE) to monitor the initial steps of sterol uptake by three independent approaches: fluorescence spectroscopy, fluorescence microscopy and sterol quantification by HPLC. Using specific fluorescence characteristics of DHE we showed that the entry of sterol molecules into plasma membrane is not spontaneous but requires assistance of two ABC (ATP-binding cassette) pumps – Aus1p or Pdr11p. DHE taken up by uptake-competent *hem1ΔAUS1PDR11* cells could be directly visualized by UV-sensitive wide field fluorescence microscopy. HPLC analysis of sterols revealed significant amounts of exogenous ergosterol and DHE (but not cholesterol) associated with uptake-deficient *hem1Δaus1Δpdr11Δ* cells. Fluorescent sterol associated with these cells did not show the characteristic emission spectrum of membrane-integrated DHE. The amount of cell-associated DHE was significantly reduced after enzymatic removal of the cell wall. Our results demonstrate that the yeast cell wall is actively involved in binding and uptake of ergosterol-like sterols.

1. Introduction

Sterols are essential membrane components of all eukaryotic cells. While cholesterol is a typical sterol in animal cells, fungi synthesize and preferentially incorporate ergosterol into their membranes. Although there are several structural differences between cholesterol and ergosterol, they both efficiently support growth of the yeast *Saccharomyces cerevisiae*. However, once taken up, their intracellular fates are diverse. The most striking difference is the lack of esterification of external ergosterol contrasting to a high accumulation of cholesteryl esters in anaerobic or heme deficient cells [1]. Thus cholesterol as an experimental probe does not necessarily reflect the behavior of ergosterol in uptake studies and results obtained using cholesterol should be interpreted with caution.

Several reports proved that dehydroergosterol (DHE), a fluorescent ergosterol analog, can be used as a probe for cholesterol in cellular studies in mammalian cells [2–4]. DHE differs from ergosterol only in one double bond at position 9 creating a system of three conjugated double bonds giving DHE its fluorescent properties. DHE induces the biologically relevant liquid-ordered phase in model membranes similar as cholesterol and ergosterol, and DHE's biophysical properties like rigidifying phospholipid membranes, resemble most closely those of ergosterol [5,6].

S. cerevisiae is regarded as a typical facultative anaerobe, however, its anaerobic growth needs to be supported by exogenous sterols and unsaturated fatty acids [7]. Interestingly, *S. cerevisiae* cannot take up sterols under aerobic conditions, even if intracellular sterols are depleted due to defective ergosterol synthesis. This phenomenon described as "aerobic sterol exclusion" [8], is related to heme-dependent regulation of the sterol uptake machinery. Sterol uptake is a complex process that can be divided into three steps: (1) interaction of external sterol with the cell wall, (2) incorporation of sterol molecules into the plasma membrane and (3) actual integration into intracellular membrane turnover. A screen for proteins regulated by uptake controlling hypoxic transcription factor Upc2/Mox4 identified two plasma membrane ABC transporters, Aus1p and Pdr11p, and one cell wall mannoprotein (Dan1p) as being involved in sterol uptake. Simultaneous deletion of *AUS1* and *PDR11* genes completely abolished sterol uptake resulting in an anaerobic lethal phenotype of the double mutant [9]. Li and Prinz [10] suggested that entry of external ergosterol into the

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plasma membrane is spontaneous and Aus1p and Pdr11p mediate specifically non-vesicular movement of plasma membrane sterol to the endoplasmic reticulum. Using three different experimental approaches we show in this report that DHE is an excellent probe for studies of sterol uptake in yeast and demonstrate that Aus1p and Pdr11p are important in the initial step of the uptake process – incorporation of sterol molecules into the plasma membrane.

2. Materials and methods

2.1. Yeast strains and growth conditions

Following *S. cerevisiae* strains were used in this study: *hem1Δ* (BY4741 MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hem1::LEU2*) and *hem1Δaus1Δpdr11Δ* (BY4741 MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 hem1::LEU2 aus1::kanMX4 pdr11::kanMX4*) [11]. Cells were grown aerobically at 30 °C in liquid YEPD media (1% yeast extract, 2% peptone and 2% glucose) supplemented with 20 μg/ml of DHE (gift from F. Schroeder, Texas A&M University, USA), ergosterol (Fluka, Switzerland) or cholesterol (Sigma, Germany) and 0.067% Tween 80 (source of oleic acid; Sigma, Germany). Unless mentioned otherwise, cells were washed twice with 0.5% Nonidet P40 (NP-40) and twice with water prior to further analysis.

2.2. Fluorescence spectroscopy of DHE

DHE-specific fluorescence was measured on LS-50B fluorescence spectrometer (Perkin–Elmer, USA). Measurements were performed in Emission Scan mode at excitation wavelength of 324 nm. The emission spectrum of DHE in various detergents was acquired at sterol concentration of 1 μg/ml. Intact cell measurements were performed in suspensions of DHE-grown cells at OD_{600} = 0.3. Emission spectra of DHE in crude membrane fractions were recorded at 5 μg protein/ml.

2.3. Removal of cell wall and isolation of crude membranes

Washed cells were pretreated by 10 mM DTT in 0.1 M Tris–H_2SO_4 (pH 9.6) and cell wall was digested by zymolyase 20T (0.33 mg/ml, Seikagaku, Japan) for 1 h at 30 °C in 1.2 M sorbitol, 10 mM KPi pH 7.5. Conversion to spheroplasts was confirmed by sensitivity to hypoosmotic conditions. For membrane isolation, spheroplasts were washed twice in 1.2 M sorbitol at 4 °C and cell wall residues were removed by subsequent treatment with lyophilized snail gut extract (0.5 mg/ml; 15 min at 30 °C), proteinase K (0.1 mg/ml; Serva, Germany) and chitinase (0.6 mg/ml; Sigma, USA) (both 20 min at 30 °C). Washed spheroplasts were suspended in cold lysis buffer (0.2 M sorbitol, 50 mM potassium acetate, 2 mM EDTA and 20 mM HEPES pH 6.8) and broken in a Dounce homogenizer at 4 °C. Unbroken cells were removed by low-speed centrifugation and crude membranes were sedimented from supernatant by centrifugation at 100,000*g* (45 min at 4 °C). Membranes were resuspended in cold lysis buffer and aliquots were used for fluorescence analysis as described above.

2.4. HPLC analysis of sterols

Non-saponifiable lipids were isolated by the modified procedure of Breivik and Owades [12]. Shortly, cells broken by homogenization with glass beads were incubated in 3 ml of 60% KOH (w/v) in 50% methanol (v/v) for 2 h at 70 °C. Non-saponifiable lipids were extracted twice with 3 ml of *n*-hexane and combined extracts were dried under N_2. Lipid residue was dissolved in acetone and analyzed by reversed phase HPLC on Agilent 1100 instrument equipped with Eclipse XDB-C8 column (Agilent Technologies, USA), diode array detector (Agilent Technologies, USA) and Corona charged aerosol detector (ESA Inc., USA). Sterols were eluted at 30 °C with 95% methanol at flow rate 1 ml/min. Peak identity was determined from the retention times of standards – DHE, ergosterol, cholesterol, lanosterol (Serva, Germany) and squalene (Sigma–Aldrich, USA) and from their characteristic spectra. Sterol quantity was calculated from calibration curves constructed for individual standards.

2.5. Fluorescence microscopy of DHE

Cells grown in the presence of DHE were washed, transferred to microscope slides, imbedded in agarose and imaged on a UV-sensitive wide field microscope Leica DMIRBE with a 63×, 1.4 NA oil immersion objective (Leica Lasertechnik GmbH, Germany) equipped with a Orca BT512 4-stage peltier and water cooled (−80 °C) CCD camera (Hamamatsu Photonics Inc., Japan) and a Lambda SC smart shutter (Sutter Instrument Co., USA), driven by ImagePro Plus (Media Cybernetics, Inc., USA). Optical components and excitation source were described previously [13]. DHE-stained or unstained yeast cells were imaged by repeated acquisition (typically 30 planes) with an acquisition time of 2 s and no lag time between the acquisitions.

2.6. Image analysis

DHE-containing cellular structures were revealed and discriminated from autofluorescence by analyzing local decay rate of the fluorescence emission of the sterol. Bleaching of DHE can be described using a mono-exponential decay function

$$f(t) = A * \exp(-k * t) + B \quad (1)$$

where A is the amplitude, k, the bleach rate constant and B, the residual fluorescence [14,15]. Estimation of DHE bleaching on a pixel-by-pixel basis was performed using PixBleach, a plugin we developed recently for the image analysis program ImageJ (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/). This analysis provides an amplitude image, a background image and a time constant map (corresponding to the parameters A, B and k in Eq. (1), above). DHE intensity was measured per yeast cell from the amplitude images using tools in ImageJ. Intensity values were exported to SigmaPlot 9.0 (SPSS Inc., USA) and plotted as histogram.

3. Results

3.1. Dehydroergosterol as a fluorescent probe in the study of yeast sterol biogenesis

To evaluate the potential of dehydroergosterol (DHE) as a fluorescent substitute of ergosterol in yeast, we studied DHE metabolism under conditions enabling the uptake of external sterols in *S. cerevisiae*. DHE was able to substitute ergosterol as growth supplement during anaerobiosis or in *hem1Δ* genetic background. Similar to ergosterol and contrasting with cholesterol, DHE taken up by *hem1Δ* cells was only poorly esterified (results not shown). The stability of internalized DHE was determined by HPLC analysis of lipid extracts of cells after long-term growth on DHE as a sterol supplement. Except for the substitution of ergosterol by DHE, sterol composition was almost identical in DHE- and ergosterol-grown cells (Supplementary Fig. S1). The usefulness of DHE as a fluorescent probe relies also in its emission characteristics in different environments. We measured emission spectra of DHE in various detergents, in uptake-competent *hem1ΔAUS1PDR11* cells loaded with DHE as well as in crude membrane fraction isolated from these cells (Fig. 1). DHE-loaded cells and crude membranes

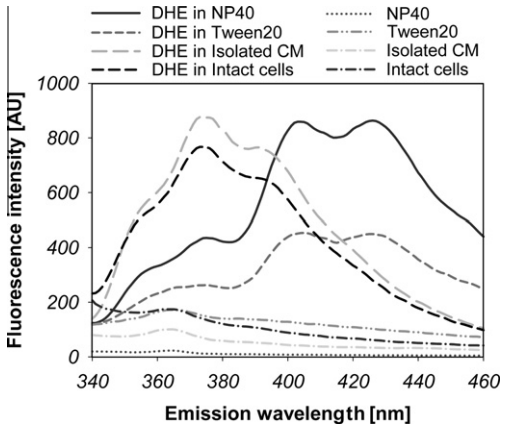

Fig. 1. Dehydroergosterol fluorescence in various environments. DHE (1 μg/ml) was dissolved in 1% NP-40 or 1% Tween-20 and emission spectra were recorded as described in Section 2. Emission spectrum of DHE in intact cells was determined in *hem1ΔAUS1PDR11* cells grown overnight in DHE-supplemented media. Emission spectrum of DHE in membranes was determined in crude membrane fraction (CM) isolated from *hem1ΔAUS1PDR11* cells grown in DHE-supplemented media as described in Section 2.

showed a specific spectrum with an emission maximum at 375 nm. This emission spectrum is characteristic for DHE in organic solvents, like chloroform and ethanol as well as for DHE in phospholipid membranes [2,16,17]. Accordingly, we conclude that the emission spectrum of DHE in the yeast cells originates from DHE monomers integrated into the cell membranes. In contrast, the emission spectrum of DHE dissolved in 1% NP40 or 1% Tween 20 is red-shifted with emission maxima at 400 and 430 nm (Fig. 1). Thus, the emission of DHE in cells and in isolated cell membranes reflects membrane-incorporated sterol without interference from DHE–detergent complexes. Taken together, our experiments proved that DHE is a suitable fluorescent probe for studying various aspects of sterol metabolism in yeast, particularly sterol uptake.

3.2. Role of Aus1p and Pdr11p in sterol uptake

The involvement of putative sterol importers, Aus1p and Pdr11p in external sterol uptake was studied in *hem1Δ* genetic background. *hem1Δ* cells are unable to synthesize ergosterol, however, their growth can be supported by external sterols. Disruption of *AUS1* and *PDR11* in these cells abolishes external sterol uptake and triple mutant *hem1Δaus1Δpdr11Δ* cease to grow after few generations despite the presence of sterols in the medium (results not shown). We estimated sterol content in *hem1ΔAUS1PDR11* cells and *hem1Δaus1Δ pdr11Δ* triple mutants cultivated on ergosterol, DHE or cholesterol by HPLC (Fig. 2A). Highest sterol levels were observed in cholesterol-grown *hem1ΔAUS1PDR11* cells which is consistent with high esterification of cholesterol compared to ergosterol and related sterol species [1]. Only negligible amounts of cholesterol were detected in extracts from *hem1Δaus1Δpdr11Δ* cells lacking sterol transport proteins (Fig. 2A, light grey bars), in accordance with previous reports [9]. Surprisingly, triple mutant cells retained high levels of ergosterol or DHE resistant to repeated washing with detergents (NP-40, Tween 20, SDS) (Fig. 2A, black and dark grey bars, respectively).

Apparently this pool of sterol molecules associated with triple mutant cells could not be utilized to support cell growth. To elucidate the localization of DHE associated with *hem1Δaus1Δpdr11Δ* mutant cells, we analyzed fluorescence of both *hem1ΔAUS1PDR11* and *hem1Δ aus1Δ pdr11Δ* cells (Fig. 2B). The characteristic emission spectrum of membranous DHE was only observed in *hem1-*

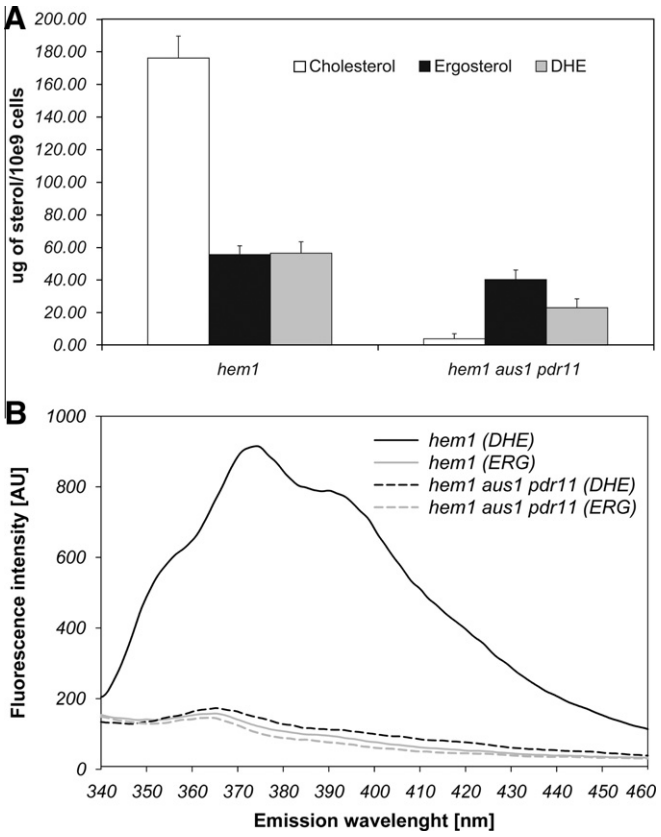

Fig. 2. Effect of *AUS1 PDR11* disruption on sterol uptake. (A) Amount of external sterols associated with sterol uptake-competent (*hem1ΔAUS1PDR11*) and sterol uptake-deficient (*hem1Δaus1Δpdr11Δ*) cells. Total sterols from cells grown for 24 h in media supplemented with corresponding sterol were quantified by HPLC using calibration curves for corresponding standards. Data are the averages of a minimum of five independent experiments ± S.E.M. (B) Fluorescence of DHE associated with *hem1ΔAUS1PDR11* and *hem1Δaus1Δpdr11Δ* cells. Emission spectra of cells grown 24 h in media supplemented with indicated sterols were measured at excitation wavelength 324 nm specific for DHE.

ΔAUS1PDR11 cells, while *hem1Δaus1Δpdr11Δ* triple mutants showed no fluorescence attributable to DHE. Since we detected significant amounts of ergosterol and DHE in triple mutants by HPLC (Fig. 2A) but measured no DHE fluorescence in intact cells (Fig. 2B), we hypothesized that fluorescence of DHE is somehow quenched in *hem1Δaus1Δpdr11Δ* cells, probably by its association with the cell wall. In line with this notion, we found that DHE fluorescence was recovered after extraction of lipids from the triple mutant grown on DHE and TLC analysis of lipid extract revealed significant amount of DHE associated with uptake-deficient *hem1-Δaus1Δpdr11Δ* cells that was not esterified by enzymes Are1p and Are2p located in the endoplasmic reticulum. This contrasts with the presence of DHE esters in uptake-competent *hem1-ΔAUS1PDR11* cells (Supplementary Fig. S2). Digestion of the cell wall with zymolyase removed up to 50% of bound ergosterol or DHE, as measured by HPLC. This indicates that significant part of ergosterol or DHE associated with *hem1Δaus1Δpdr11Δ* cells is bound to the cell wall.

3.3. Fluorescence microscopy imaging of DHE grown cells

As a third approach to assess uptake of external DHE in yeast cells, we used quantitative UV-sensitive wide field fluorescence microscopy (Fig. 3). For these experiments, we used the same protocol for cell labeling with DHE as for HPLC, TLC and spectrofluorimetry. Uptake-competent *hem1ΔAUS1PDR11* cells were labeled

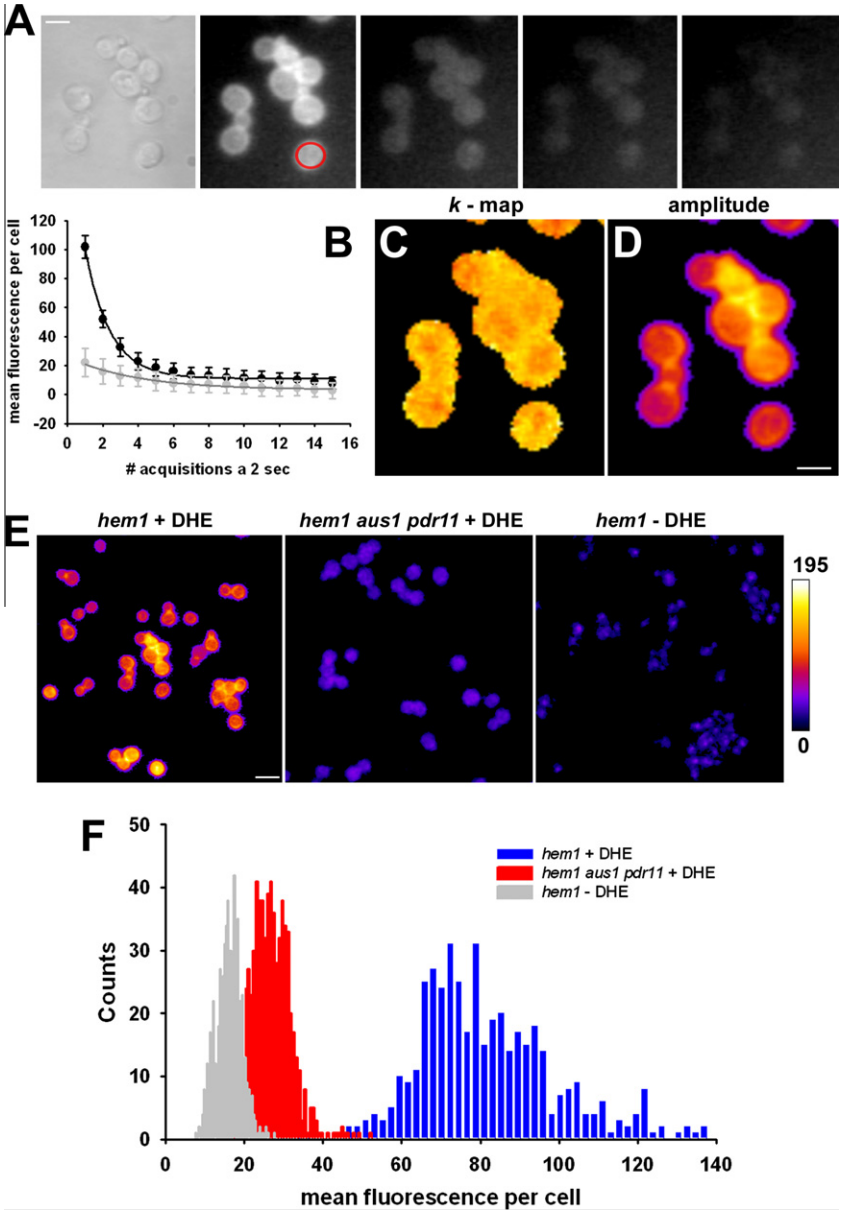

Fig. 3. Direct visualization of dehydroergosterol uptake in *hem1Δ* and *hem1Δaus1Δpdr11Δ* cells by UV-sensitive wide field microscopy. Yeast strains grown in the presence or absence of DHE for 24 h were analyzed by fluorescence microscopy as described in Section 2. Cells were repeatedly imaged with an acquisition time of 2 s to get an image stack containing all fluorescence emitted by DHE. (A) Left-to-right: differential interference contrast DIC image and images of fluorescence DHE image stack in the 1st, 3rd, 5th and 10th plane of the *hem1ΔAUS1PDR11* strain, respectively. The fading intensity indicates rapid DHE bleaching during continuous illumination. (B) Time course of intensity decay in *hem1ΔAUS1PDR11* cells incubated with DHE (black symbols, data; black line, fit) or without DHE (grey symbols, data; grey line, fit). (C and D) Pixel-wise bleach rate fitting of the same function to the image stack from panel A provides a rate constant map (C 'k-map') and an amplitude image (D). The amplitude represents bleaching intensity in the UV. (E) Amplitude image for *hem1ΔAUS1PDR11* cells + DHE (left), *hem1Δaus1Δpdr11Δ* cells + DHE (middle) and *hem1* cells incubated with ergosterol (right). (F) Mean fluorescence from the amplitude image for these three conditions presented as histogram. At least 450 cells per condition from three separate experiments were used for the analysis.

with DHE mostly at the cell periphery, which is consistent with predominant localization of free sterols in the yeast plasma membrane. We showed previously that DHE bleaches rapidly in a mono-exponential process in cell and model membranes, with a characteristic rate constant under our acquisition conditions of k ~0.28–0.35 s^{-1} [15]. Thus, a photobleaching analysis was used to verify that the observed fluorescence originates from emission of membrane-inserted DHE. Cell-associated fluorescence in uptake-competent *hem1ΔAUS1PDR11* cells experienced a rapid decay in response to repeated illumination of the field (Fig. 3A and B). Non-labeled cells had low autofluorescence that decreased only slightly during repeated illumination of the field. A mono-exponential fit according to Eq. (1) (Section 2) to the bleaching-induced intensity decay of selected yeast cells gave a bleach rate constant of $k = 0.36\ s^{-1}$ for DHE-labeled *hem1ΔAUS1PDR11* cells (Fig. 3B). The same analysis for non-labeled cells gave $k = 0.13\ s^{-1}$. A pixel-wise analysis of DHE bleaching kinetics provides a time constant map characterizing the kinetics of the bleaching process (Fig. 3C), an amplitude image giving the intensity of the bleaching fluorophore fraction in the first image (3D) and a background image resembling residual autofluorescence (not shown). The amplitude maps were used for further analysis of DHE uptake. While uptake-competent *hem1ΔAUS1PDR11* cells were intensively stained with DHE, *hem1Δaus1Δpdr11Δ* showed only faint fluorescence slightly above the background autofluorescence of *hem1ΔAUS1PDR11* cells grown on ergosterol (Fig. 3E and

F). A quantitative analysis revealed that *hem1ΔAUS1PDR11* cells have approximately 6.3-fold more DHE fluorescence intensity per cell than the triple mutants, which again proved the key role of Aus1p and Pdr11p in the entry of external sterols into the plasma membrane.

4. Discussion

The reliability of fluorescent sterol probes in cell biology is usually limited by their structural dissimilarity to the native molecule. It has been shown both in cell and model membranes, that sterol probes with attached fluorophore, like nitrobenzoxadiazol (NBD)–cholesterol, have different properties than cholesterol itself [2,5]. In contrast to such sterol probes, DHE with its intrinsic fluorescence has been shown to resemble cholesterol and ergosterol in model membranes and intracellular trafficking [2–4]. DHE is an ideal ergosterol analog, since it differs from ergosterol only by one double bond. As expected based on this structural similarity, we found that DHE is a particularly suitable substitute for ergosterol in studies of sterol distribution and trafficking in the yeast *S. cerevisiae*. Both fluorescence microscopy and fluorescence spectroscopy are available for *in vivo* DHE detection. Although direct visualization of DHE in cells by fluorescence microscopy has several limitations (e.g. excitation and emission wavelengths of DHE in the near UV region, relatively low fluorescence intensity and high bleaching rate), we were able to overcome these limitations and to visualize DHE taken up by yeast cells using UV-sensitive wide field fluorescence microscopy and image processing [14,15].

Our results obtained by two fluorescence-based approaches (spectroscopy and microscopy) combined with direct quantification of DHE by HPLC confirmed that two ABC pumps, Aus1p and Pdr11p, are involved in the uptake of external sterol molecules. In contrast to a previous report arguing for involvement of Aus1p and Pdr11p in non-vesicular sterol trafficking from the plasma membrane to the endoplasmic reticulum [10], our data strongly suggest that these two proteins are also required in the initial insertion of external sterol molecules into the plasma membrane. Emission spectrum of DHE taken up by *hem1ΔAUS1PDR11* is typical for DHE incorporated into membranes (Figs. 1 and 2B), while this spectrum is absent in uptake-deficient *hem1Δaus1Δpdr11Δ* mutants. Similarly UV-sensitive wide field fluorescence microscopy has shown clear labeling of the cell periphery with DHE in uptake-competent cells while uptake-deficient cells cultivated on DHE were only faintly labeled, slightly above the background fluorescence (Fig. 3E and F). This extremely low cell-associated fluorescence of DHE contrasted with the estimation of DHE by HPLC where relatively high levels of DHE and ergosterol (and only minute amounts of cholesterol) were found to be associated with uptake-deficient cells unable to grow in the presence of external sterols (Fig. 2A). DHE and ergosterol were sensitive to cell wall digestion by zymolyase indicating the involvement of the cell wall in sterol binding. The association of DHE with *hem1Δaus1Δpdr11Δ* cells and simultaneous absence of fluorescence indicates quenching of DHE by some unidentified component of the yeast cell wall. Extraction of lipids from these cells released DHE from quenching as confirmed by DHE-specific fluorescence of lipid extracts and TLC chromatograms (Supplementary Fig. S2). To our knowledge, no cellular components with DHE quenching capacity have been identified so far, although artificial quenchers of DHE were described [18,19]. In principle, two quenching mechanisms are possible: dynamic and static quenching [20]. For dynamic quenching, DHE quantum yield is lowered due to its collision in the excited state with a mobile quencher. A lowering of the fluorescence quantum yield would accelerate DHE bleaching kinetics, but this was not observed for the weak residual signal in the triple mutants. Based on this observation, a dynamic quenching of excited DHE molecules is unlikely. Alternatively, DHE could bind to a ground-state quencher, which would prevent most DHE molecules from getting excited by the incident UV light. At the moment it is unclear whether binding and quenching is mediated by the same cellular component. This binding is specific to *hem1Δ* (anaerobic) cell wall since no sterol binding was observed in aerobic cells (our unpublished data). The involvement of cell wall in sterol uptake is not surprising. The cell wall protein Dan1p has been shown to directly affect sterol uptake [9,21]. Moreover, expression of a group of cell wall mannoproteins, (including Dan1p) is co-regulated by Upc2/Mox4 transcription factor [22] that also regulates the expression of Aus1p and Pdr11p. Uptake of sterol may thus be linked to the cell wall on regulatory as well as on mechanistic level. We can speculate that anaerobic cell wall components serve as substrate-binding proteins (SBPs) bringing sterol molecules close to the plasma membrane – the location of Aus1p and Pdr11p [10]. This would resemble uptake of nutrients in prokaryotes, where SBPs are important for the action of ABC transporters [23].

In summary, our results show that two ABC proteins, Aus1p and Pdr11p, are required for the entry of external sterol molecules into yeast plasma membrane. The discrepancy with the results of Li and Prinz [10] who suggested the involvement of these pumps in the non-vesicular transport of sterols from plasma membrane to endoplasmic reticulum may be explained by the inability of their experimental setup to discriminate between cell wall-associated and membrane-integrated sterols. Several eukaryotic ABC proteins were described to stimulate transmembrane transport of lipid-like molecules but all of them seem to "pump" their substrates in the outward direction. To our knowledge, only few eukaryotic ABC pumps have been reported to be involved in substrate transport in the inward direction [24–27]. The precise mechanism of sterol uptake and involvement of Aus1p and Pdr11p, namely whether these proteins are actual sterol importers, requires further functional studies, like reconstitution of Aus1 and Pdr11 into model membranes.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.099.

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