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Functional characterization of the *Saccharomyces cerevisiae* ABC-transporter Yor1p overexpressed in plasma membranes

Ioana Grigoras [1], Myriam Lazard *, Pierre Plateau, Sylvain Blanquet

Laboratoire de Biochimie, Ecole Polytechnique, CNRS, F-91128 Palaiseau cedex, France

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Abstract

Yor1p, a *Saccharomyces cerevisiae* plasma membrane ABC-transporter, is associated to oligomycin resistance and to rhodamine B transport. Here, by using the overexpressing strain Superyor [A. Decottignies, A.M. Grant, J.W. Nichols, H. de Wet, D.B. McIntosh, A. Goffeau, ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p, J. Biol. Chem. 273 (1998) 12612–12622], we show that Yor1p also confers resistance to rhodamine 6G and to doxorubicin. In addition, Yor1p protects cells, although weakly, against tetracycline, verapamil, eosin Y and ethidium bromide. The basal ATPase activity of the overexpressed form of Yor1p was studied in membrane preparations. This activity is quenched upon addition of micromolar amounts of vanadate. V_{max} and K_m values of $\sim 0.8\ s^{-1}$ and $50 \pm 8\ \mu M$ are measured. Mutations of essential residues in the nucleotide binding domain 2 reduces the activity to that measured with a Δ*yor1* strain. ATP hydrolysis is strongly inhibited by the addition of potential substrates of the transporter. Covalent reaction of 8-azido-[α-^{32}P]ATP with Yor1p is not sensitive to the presence of excess oligomycin. Thus, competition of the drug with ATP binding is unlikely. Finally, we inspect possible hypotheses accounting for substrate inhibition, rather than stimulation, of ATP hydrolysis by the membrane preparation.

Keywords: Yor1p; ABC transporter; Plasma membranes; ATPase activity

1. Introduction

Most ATP-binding cassette (ABC) proteins mediate ATP-driven selective transport of a wide range of substrates across biological membranes [1–3]. In accordance with their common function, ABC-transporters share a conserved structural architecture consisting of two homologous halves, each containing a transmembrane domain (TMD) and a nucleotide binding domain (NBD). The TMDs harbor the substrate binding sites and the NBDs couple the energy of ATP hydrolysis to transport. The NBDs share extensive amino acid sequence identities and characteristic motifs, the Walker A and Walker B, found in numerous nucleotide binding proteins [4]. Another motif called the C-loop or signature sequence defines this family [5]. In addition to these motifs, sequence alignments have highlighted very conserved residues that define the A-, Q-, D-, and H-loops [6–8]. Resolution of the crystal structures of various isolated NBDs and of full transporters shows that the functional unit is a "head to tail" dimer in which one molecule of ATP is sandwiched between the Walker A motif in one monomer and the signature in the other. The other ATP substrate interacts with the Walker A motif in the second monomer and the signature of the first monomer (for review see [8]).

ABC-proteins have been divided into subfamilies based on similarities in overall protein primary structures, including relative order of the NBDs and TMDs and amino acid sequence homologies. Seven mammalian subfamilies have been distinguished [9]. The ABCC subfamily includes 13 proteins of great clinical importance. The CFTR (ABCC7) protein is a chloride

Abbreviations: ABC, ATP binding cassette; TMD, Transmembrane domain; NBD, Nucleotide binding domain; P-gp, P-glycoprotein; CFTR, Cystic fibrosis transmembrane conductance regulator; MRP, Multidrug resistance associated protein; p-CMPS, p-chloromercuriphenylsulfonate; DCC, *N,N′*-di-cyclohexyl-carbodiimide; NEM, N-ethylmaleimide; DTT, 1–4 dithiothreitol; BSA, Bovine serum albumine; EDTA, Ethylenediaminetetraacetic acid; PNK, Polynucleotide 5′-hydroxyl-kinase; MIC, Minimal inhibitory concentration

* Corresponding author. Tel.: +33 1 69 33 48 85; fax: +33 1 69 33 49 09.
E-mail address: mimi@botrytis.polytechnique.fr (M. Lazard).
[1] Current address: Institut des Sciences du Végétal, CNRS, Avenue de la Terrasse, Bât. 23, F-91198 Gif sur Yvette cedex, France.

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ion channel and mutations in CFTR cause cystic fibrosis. The ABCC8 and ABCC9 proteins bind sulfonylurea and regulate potassium channels involved in modulating insulin secretion. The rest of the subfamily is composed of nine or probably ten multidrug resistance-related genes. Of these, ABCC1 (or MRP1) has been extensively studied because it is involved in cancer cell resistance to chemotherapy [10].

In the yeast *Saccharomyces cerevisiae*, six proteins, homologous to the ABCC human subfamily members, compose subclass II.1 [6]. The most studied protein in this subclass is the yeast cadmium factor (Ycf1p), functionally homologous to MRP1. Ycf1p is a vacuolar transporter active in heavy metal and drug detoxification through conjugation to glutathione (GSH) [11,12]. The structural and functional similarities between the human and yeast ABCC subfamilies suggest that the latter may provide useful models to study this particular class of ABC-transporters.

In the yeast subclass II.1, Yor1p is a plasma membrane ABC-protein involved in the resistance to oligomycin and to organic anions such as rhodamine B [13,14]. Transport of these drugs is obtained at the expense of ATP. Possible Yor1p UTPase activity has been searched for by Decottignies et al. [15], but without success. Expression of Yor1p is up-regulated by the transcription factors Pdr1p and Pdr3p. Decottignies et al. [15] have shown that, in a Pdr1–3 gain of function mutant, *YOR1* placed under the control of the *PDR5* promoter was constitutively overexpressed. Under this condition, the *YOR1* product reached up to 10% of total membrane proteins. This property was used to isolate partially purified protein. In the present study, we used the expression system described above to further investigate the properties of Yor1p. We have chosen to study the behaviour of plasma membrane extracts in order to maintain over-expressed Yor1p in a native environment.

2. Materials and methods

2.1. Materials

Oligomycin, rhodamine B, rhodamine 6G, doxorubicin, verapamil, tetracycline, fluorescein, aprotinin, leupeptin, pepstatin, benzamidine, ovomucoid, p-chloromercuriphenylsulfonate (p-CMPS), *N,N'*-di-cyclohexylcarbodiimide (DCC), N-ethylmaleimide (NEM), glass beads (acid-washed, 425–600 μm) and G418 were purchased from Sigma. Sodium orthovanadate was from Acros Organics. Rhodamine 101, heparin, ouabain, antipain, chymostatin and o-phenanthroline were from Fluka. Sodium azide and erythrosin B were from Merck. The "HMW-SDS Calibration Kit" and ATP were from Amersham Biosciences. [γ-^{32}P]ATP was from Perkin Elmer. 8-azido-[α-^{32}P]ATP was from MP Biomedicals.

All the oligonucleotides used for gene constructions are listed in Table 1. Plasmid pSK-PDR5PPUS is described in [15]. pBluescript SK+ and pYES2 were from Stratagene and Invitrogen, respectively.

2.2. Strains and growth media

The *S. cerevisiae* strain Superyor *(*MATα, *PDR1-3, his1, Δyor1*::*hisG, Δsnq2*::*hisG, Δpdr5*::*PDR5PROM-YOR1-PDR5STOP, Δpdr10*::*hisG, Δpdr11*::*hisG, Δycf1*::*hisG, Δpdr3*::*hisG)* is described in [15]. Cells were grown at 30 °C in rich YT medium, containing 1% yeast extract (Difco), 1% Bacto-tryptone (Difco), and either 2% glucose (YT-glucose medium) or 2% glycerol (YT-glycerol medium), as indicated. Transformations of yeast cells were performed by the lithium acetate method [16]. Transformants were plated on YT-glucose plates containing 200 μg/ml G418 to select the Kan^R marker.

2.3. Construction of the S. cerevisiae strains

2.3.1. Construction of Superyor-Δyor

Disruption of *YOR1* was achieved by insertion of the Kan^R selectable marker between nucleotides 1751 and 4311 of the *YOR1* open reading frame.

Table 1
Oligonucleotides used in this study

Oligonucleotide	Sequence
LINKERY1	AATTGAGCTCACTAGTGGCCTTAAAGGCCCTCGAGGCATGCCCCGGGATGCAT
LINKERY2	CTAGATGCATCCCGGGGCATGCCTCGAGGGCCTTTAAGGCCACTAGTGAGCTC
CYC1DEB	CCGGAATTCGCATGCGGGCCGCATCATGTAATTAGTTATG
CYC1FIN	CGCGGATCCATGCATGGCCGCAAATTAAAGCCTTCGAGCG
YOR1-NOT	GGGGCGGCCGCTACATCCTCAGTGGACTAC
YOR2	GCTCTAGAATTAGTGATGGTGATGGTGATGCGATCCTCTGTTCTCGAAATCATT
YOR3	CATCACCATCACCATCACTAATTCTAGAGCGTACGCTGCAGGTCGACG
YOR4	TGAATGAAATGACAAGCAGACATCGATGAATTCGAGCTCG
YOR5	CGAGCTCGAATTCATCGATGTCTGCTTGTCATTTCATTCA
YOR6-XHO	CGCTCGAGCCAACAGAGCCTGAATGAA
YOR-EXT-1	CTAGGTCTGGTATTGTGGAAAATGATTTCG
YOR-EXT-2	AGAACAGAGGATCGCATCACCATCACCATC
YOR-EXT-3	ACCATCACCATCACTAATA
YOR-EXT-4	CTAGTATTAGTGATGGTGATGGTGATGGTGATG
YOR-EXT-5	GTGATGCGATCCTCTGTTCTCGAAATCATT
YOR-EXT-6	TTCCACAATACCAGAC
YOR-NOT1	GGGGCGGCCGCGATTCGCAAGAACTTAGATCC
YOR-XBA-XHO	GCCTCGAGTCTAGAATTAGTGATGGTGATGGTGATGGTGATGGTGATGCGATCCTCTGTTCTCGAAATC
YORINACT1	GTAAGGATACATGGGGTAAGCCATCTGCAAGTACTAATAAGGCGAAAAGATTGGCGTACGCTGCAGGTC
YORINACT4	TTCTTGACTAAACAACGTCCATGGTGTATCGAATTCTGCGACTTCACCCTTCTCATCGATGAATTCGAG
YNBD2MUTEQ1	AAAAATATTGATTTTGGATCAGGCTACATCCTCAGTGGA
YNBD2MUTEQ2	TCCACTGAGGATGTAGCCTGATCCAAAATCAATATTTTT
YNBD2MUTHR1	ACAATTTTGTGTATTGCTCGTAGACTGAAGACCATTGTA
YNBD2MUTHR2	TACAATGGTCTTCAGTCTACGAGCAATACACAAAATTGT

Oligonucleotides YORINACT1 and YORINACT4 (Table 1), containing 54 bases homologous to *YOR1* and 15 bases to the marker gene, were used to amplify the Kan^R cassette from plasmid pFA6a-kanMX4 [17]. The PCR product was used to transform the *S. cerevisiae* strain Superyor. The transformants were selected on YT-glucose plates containing 200 μg/ml G418 and disruption was confirmed by PCR analysis.

2.3.2. Construction of Superyor-(His)$_{10}$

Plasmid pSK-PDR5PPUS was modified to generate plasmid pSK-PDR5PPΔΔLT as follows: the unique *Xho*I and *Sac*I sites were first disrupted, then a *Sac*I–*Spe*I–*Sfi*I–*Xho*I–*Sph*I–*Sma*I–*Mlu*I linker was inserted between the *Eco*RI and the *Spe*I sites by using oligonucleotides LINKERY1 and LINKERY2. Last, the *CYC1* transcription terminator was amplified by PCR using plasmid pYES2 as template and oligonucleotides CYC1DEB and CYC1FIN as primers, and inserted between the *Sma*I and *Mlu*I sites. A cassette containing the 3′-end of the *YOR1* ORF fused to an Arg–Gly–Ser–(His)$_6$ sequence, followed by the Kan^R marker and the end of the *PDR5* ORF, was constructed as follows. First, three overlapping PCR fragments were generated : (i) the last 300 bp of the *YOR1* ORF were inserted between the *Hin*dIII and *Xho*I sites of plasmid pBluescript SK+, to give pBS YORCF5′. The latter plasmid was used as template for a PCR amplification using the YOR1-NOT and YOR2 oligonucleotide primers; (ii) the plasmid pFA6a-kanMX4 was used to amplify the *Kan^R* gene using the YOR3 and YOR4 oligonucleotide primers; (iii) the plasmid pSK-PDR5PPΔΔLT and the YOR5 and YOR6-XHO oligonucleotide primers were used to amplify the last 177 bp of the *PDR5* ORF. Then, to assemble the YOR1-(His)$_6$-Kan^R-PDR5 cassette, approximately 50 ng of each of the three fragments were combined in a PCR reaction mixture and cycled 5 times to join the fragments. Primers YOR1-NOT and YOR6-XHO were then added and amplification proceeded for 26 cycles. The PCR fragment (1987 bp) was cut by *Not*I and *Xho*I and inserted into pBluescript SK+. The sequence of the resulting *YOR1-(His)$_6$* ORF was verified. To construct strain Superyor-(His)$_{10}$, we took advantage of the presence, in the plasmid pBluescript SK+ YOR1-(His)$_6$-Kan^R-PDR5, of a 67 bp *Xba*I–*Xba*I fragment overlapping the stop codon of the *YOR1-(His)$_6$* ORF. To modify the C-terminus of Yor1p-(His)$_6$, we replaced this fragment by another generated by assembly of 6 overlapping oligonucleotides as follows: 50 pmol of each YOR-EXT-2, YOR-EXT-3, YOR-EXT-5 and YOR-EXT-6 were first 5′-phosphorylated using polynucleotide 5′-hydroxyl-kinase (PNK, Roche) for 1 h at 37 °C. After inactivation of PNK for 10 min at 70 °C, 50 pmol of each YOR-EXT-1 and YOR-EXT-4 were added and the oligonucleotide mixture was denatured at 95 °C for 2 min. The mixture was then incubated for 2 h at 37 °C with 2 units of T4 DNA ligase (Invitrogen). The resulting DNA fragment was ligated with *Xba*I-cut pBluescript SK+ YOR1-(His)$_6$-Kan^R-PDR5 and the DNA sequence of the produced plasmid was verified. The *Xho*I–*Not*I linearized pBluescript SK+ YOR1-(His)$_{10}$-Kan^R-PDR5 was used to transform the *S. cerevisiae* strain Superyor [15]. The transformants were selected on YT-glucose plates containing 200 μg/ml G418. Replacement at the *PDR5* locus of *YOR1* by the *YOR1-(His)$_{10}$* construct was verified by PCR analysis. In addition to the tag, the sequence of Yor1p-(His)$_{10}$ only differs from that of native Yor1p by the insertion of a glycine residue between the last C-terminal two residues. The C-terminus is Arg–Ser for the native protein and Arg–Gly–Ser–(His)$_{10}$ for Yor1p-(His)$_{10}$.

2.3.3. Construction of Superyor-(His)$_{10}$-H1423R and -E1392Q

The complete *YOR1* ORF was inserted in multicopy plasmid pYES2 by the gap repair method [18] to give pYOR. The latter plasmid was used as template, with oligonucleotides YOR-NOT1 and YOR-XBA-XHO as primers, to generate a PCR fragment containing the last 520 bp of the *YOR1* ORF plus the RGS-(His)$_{10}$ coding sequence. This fragment was cut by *Not*I and *Xho*I and inserted into pBluescript SK+ to give pBluescript SK+ YOR-NX1. The sequence of the inserted fragment was verified. Site-directed mutagenesis were achieved in one step using pBluescript SK+ YOR-NX1 as template and a couple of mutagenic primers, in PCR reactions using Pfu Turbo DNA polymerase (Stratagene). To introduce the E1392Q and H1423R mutations, primers YNBD2MUTEQ1/2 and YNBD2MUTHR1/2 were used, respectively. After thermal cycling, methylated wild-type DNA was restricted in the presence of *Dpn*I before transformation of XL1-Blue cells (Stratagene). The sequence of the whole mutated fragment was verified in each case. Mutant plasmids were cut by *Not*I and *Xba*I, and the insert was ligated with *Not*I/*Xba*I restricted pBluescript SK+ YOR1-(His)$_6$-Kan^R-PDR5. It should be noted that the internal *Xba*I site is protected by methylation and is, therefore, resistant to restriction when the latter plasmid is produced in a *dam*$^+$ strain. Plasmids pBluescript SK+ YOR1-(His)$_{10}$-E1392Q-Kan^R-PDR5 and YOR1-(His)$_{10}$-H1423R-Kan^R-PDR5 digested by *Xho*I and *Not*I were used to transform the *S. cerevisiae* strain Superyor. The transformants were selected as above.

2.4. Drug resistance assays

Cells grown at 30 °C in YT-glucose medium were harvested in the exponential growth phase, washed and diluted in water to obtain samples with optical densities at 650 nm of 1, 0.1 and 0.01. To determine minimal inhibitory concentrations (MICs), 5 μl of each sample were spotted onto YT-glucose plates containing increasing concentrations of the various studied drugs (Table 2), or onto YT-glycerol plates containing increasing concentrations of oligomycin. The plates were analysed after 2 days of incubation at 30 °C. By definition, an MIC value, as shown in Table 2, is the concentration of the drug for which the cells do not grow any more. MIC regions for each drug were first searched for by using large drug concentration scales. Next, to refine the MIC value, closer concentrations of drugs were used. We arranged to assay concentrations around the MIC that did not differ by a factor greater than 2. Moreover, with each drug, the experiments were reproducibly performed at least three times. Thus, if a MIC value was possibly overestimated, the error cannot exceed 50% of the indicated MIC value.

2.5. Membrane preparation

The *S. cerevisiae* cells were grown at 30 °C in YT-glucose medium, harvested in early stationary growth phase and washed in buffer A (50 mM Tris–HCl (pH 8.0), 10% glycerol, 10 mM 2-mercaptoethanol). The pellets were resuspended at 0.5 g of cells per ml in buffer A supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml antipain, 0.5 μg/ml chymostatin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin A, 100 μg/ml benzamidine, 1 μg/ml o-phenanthroline and 6 μg/ml ovomucoid) and broken with an equal volume of glass beads by vortexing the cells 10×30 s,

Table 2
Effect of different compounds on the growth of the *S. cerevisiae* strains Superyor and Superyor-Δyor

	Minimal inhibitory concentration (MIC)		
	Superyor-Δyor	Superyor	Resistance factor
Oligomycin	0.3 μM	>100 μM	>300
Rhodamine B	200 μM	7.5 mM	37.5
Rhodamine 6G	1 μM	200 μM	200
Rhodamine 101	0.25 mM	1 mM	4
Erythrosin B	2 mM	15 mM	7.5
Eosin Y	10 mM	20 mM	2
Fluorescein	30 mM	30 mM	1
Doxorubicin	25 μM	250 μM	10
Tetracycline	10 mM	>20 mM	>2
Verapamil	0.8 mM	2 mM	2.5
o-Phenanthroline	75 μM	75 μM	1
Ethidium bromide	50 μM	75 μM	1.5
$CdCl_2$	30 μM	30 μM	1

Cells were grown at 30 °C in liquid YT-glucose medium, harvested in the exponential growth phase, washed and diluted in water, to obtain samples with optical densities at 650 nm of 1, 0.1 and 0.01, respectively. 5 μl of each sample were spotted onto YT-glucose plates containing increasing concentrations of the compounds under study and left to incubate at 30 °C for 2 days (see Fig. 1). In the case of oligomycin, YT-glycerol plates were used. The MIC for a drug is defined as the minimal concentration at which no more growth is observed at the highest concentration of cells. Results of three independent experiments were in agreement. Screened concentrations around the MIC value did not differ by a factor more than 2. Thus, if a MIC value was possibly overestimated, the error cannot exceed 50% of the indicated value (see Materials and methods).

interrupted by 30 s on ice. Debris were removed by a low speed centrifugation (2000×*g* for 3 min) and the membrane pellet (20,000×*g* for 60 min) was resuspended at 5 mg proteins/ml in buffer A containing 1 M NaCl plus the full set of protease inhibitors. After centrifugation at 20,000×*g* for 60 min, the pellet was resuspended in buffer A.

2.6. ATPase assay

Routinely, initial rates of ATPase activity from 3 μg membrane proteins were assayed in a final volume of 100 μl at 37 °C in 50 mM Tris–HCl (pH 7.5) containing 2 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (200 Ci/mol), 0.2 mM $(NH_4)_6Mo_7O_{24}$, 50 mM KNO_3, 5 mM NaN_3, 10 μM EDTA, 2% glycerol and 0.2 mM DTT. The reactions were quenched by the addition of 1 ml of a 1.2% (w/v) suspension of activated charcoal in 50 mM sodium acetate (pH 4.5) plus 0.35% (w/w) perchloric acid. After 5 min of incubation on ice to allow ATP binding to charcoal, the supernatant was recovered by two successive centrifugations at 20,000×*g*, for 3 min each. 750 μl of the supernatant containing the released γ-^{32}P were mixed with 5 ml of scintillation fluid (Pico-Fluor 15 from Perkin Elmer) and quantified using the LS 6500 Multi-Purpose Scintillation Counter (Beckman).

To determine the K_m and V_{max} values for ATP, we measured initial rate of P_i production as a function of ATP concentration in the assay. The conditions were as above, except that magnesium was 4 mM. In each assay, the amount of [γ-^{32}P] ATP was fixed at 200 nCi (i. e. ~440,000 dpm). ATP was varied from 10 μM to 2 mM. The reason why we did not maintain constant the specific activity of ATP is as follows: radioactive ATP was only ~97–98% pure according to Perkin Elmer. As a result, we measured a background signal of ~6000–10,000 dpm. Such a background corresponds to radioactivity that is not retained on charcoal even in the absence of any incubation. Thus, upon an increase in specific radioactivity at a given total ATP concentration, both the signal and the background increased in the same ratios, and the accuracy of the assay was not improved. Under our conditions, at 2 mM ATP, the measured overall signal was 16,000 dpm in 10 min with a background signal of 6000 dpm.

K_m and V_{max} values were derived from iterative non-linear fits of the theoretical Michaelis equation to the experimental values, using the Levenberg–Marquardt algorithm as previously described [19]. Note that in the used procedure, accuracies on each measurement are taken into account in the calculation.

2.7. Protein analysis

SDS-PAGE analysis was carried out in 10% polyacrylamide gels using the Bio-Rad Mini-Protean II electrophoresis cell. Before loading on the gel, samples were diluted in sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% SDS, 5% glycerol, 20 mM 2-mercaptoethanol and 0.5 mg/ml bromophenol blue) and incubated at 37 °C for 20 min. Protein concentration was determined by the Bradford assay, using the Bio-Rad Protein Assay kit with BSA as the standard, in the presence of 0.1% (v /v) Triton X-100.

2.8. Binding of 8-azido-[α-^{32}P]ATP

Membranes (10 μg of protein) were incubated for 20 min in a final volume of 20 μl at 4 °C in 50 mM Tris–HCl (pH 7.5), containing 2 mM $MgCl_2$, 10% glycerol and the indicated concentration of 8-azido-[α-^{32}P]ATP (20 Ci/mmol). The samples were then illuminated with a UV lamp (312 nm, 6×15 W) for 10 s, dissolved in sample buffer and analysed by SDS-PAGE. After electrophoresis, gels were colored with Coomassie blue R250, dried and autoradiographed.

3. Results

3.1. Drug resistance profile of overexpressed Yor1p

To investigate the drug resistance properties that may be assigned to Yor1p, we took advantage of the overproduction of Yor1p by the strain Superyor [15] in a context where the genes of at least 5 major ABC drug transporters (*PDR5, YCF1, SNQ2, PDR10, PDR11*) are deleted. A Superyor-Δyor strain was also produced by inserting a geneticin resistance cassette in the chromosome of Superyor cells. The resulting Yor1 protein is interrupted at residue 583, just inside NBD1. Drug resistance was assayed by comparing the growth of Yor1p overexpressing cells to that of Δ*yor1* cells spotted on plates containing increasing concentrations of the compound under study. Fig. 1 confirms that Yor1p is involved in oligomycin and rhodamine B resistance. Cells overexpressing Yor1p continued to grow at concentrations of oligomycin and of rhodamine B over 100 μM and 5 mM, respectively. In contrast, Δ*yor1* cells stopped growing at 0.3 μM oligomycin and 0.2 mM rhodamine B. We also investigated the susceptibility of Superyor cells to analogues of rhodamine B and to drugs towards which a Δ*yor1* strain had previously displayed increased sensitivity [20]. Chemical formulas of these molecules are shown in Fig. 2. Overexpression of Yor1p conferred increased resistance to rhodamine 6G and to rhodamine 101 (Table 2). With rhodamine 6G, the factor of increase was of the same order of magnitude as

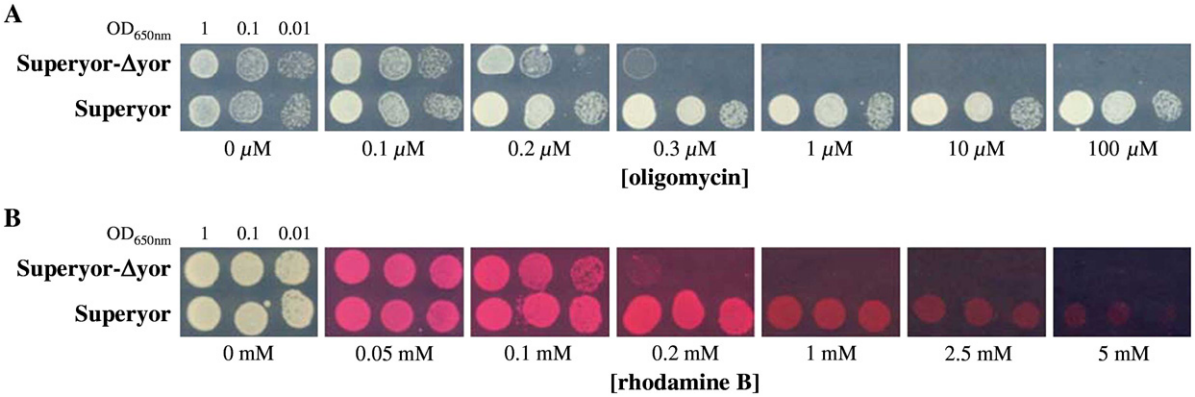

Fig. 1. Effect of oligomycin (A) and rhodamine B (B) on the growth of *S. cerevisiae* strains Superyor and Superyor-Δyor. Cells were grown at 30 °C in liquid YT-glucose medium, harvested in the exponential growth phase, washed and diluted in water, to obtain samples with optical densities at 650 nm of 1, 0.1 and 0.01, respectively. Samples (5 μl) were spotted either onto YT-glucose plates containing increasing concentrations of rhodamine B (B), or onto YT-glycerol plates with increasing concentrations of oligomycin (A). The plates were left to incubate at 30 °C for 2 days.

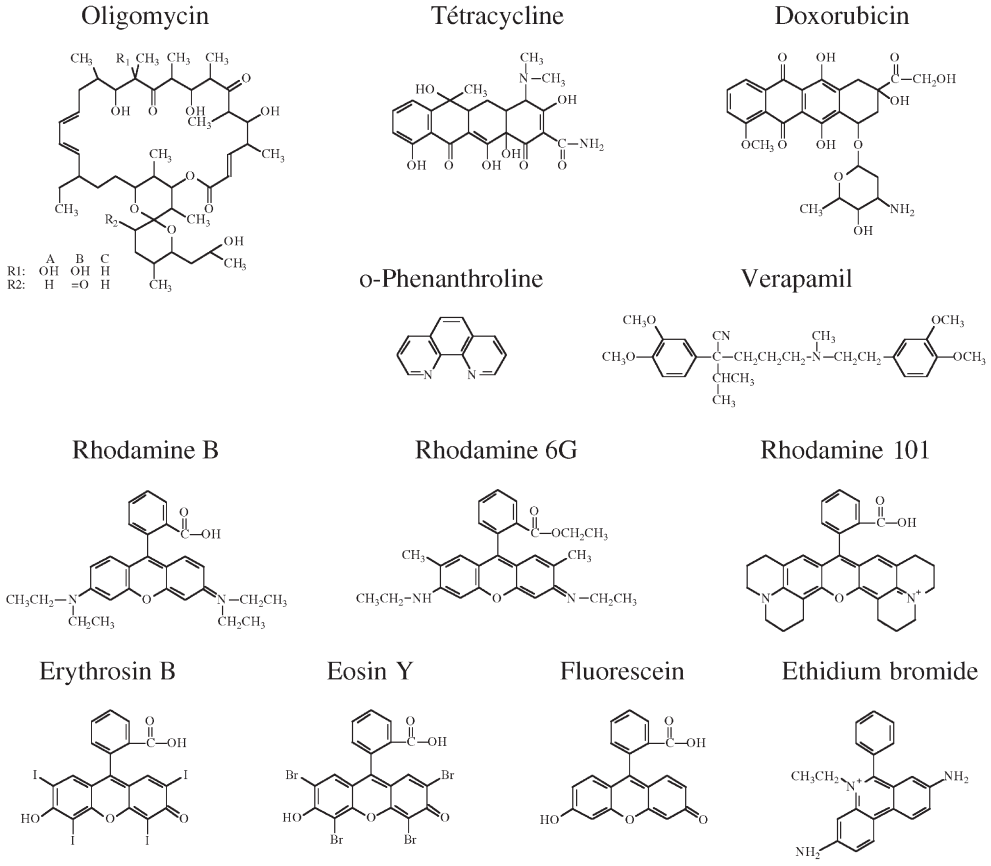

Fig. 2. Chemical formulas of the various compounds assayed for drug resistance conferred by Yor1p.

that observed with oligomycin (over 100-fold). We know that rhodamine 6G is actively extruded from intact yeast cells by Pdr5p [21]. Thus, since *PDR5* is deleted in the Superyor strain, we may safely conclude that resistance to rhodamine 6G also involves Yor1p. Yor1p overexpressing cells showed increased resistance to rhodamine analogues such as erythrosin B. In agreement with the previous report that Δ*yor1* cells are sensitive to doxorubicin and tetracycline [20], we observed increased resistance to these drugs of cells overexpressing Yor1p. We also found that Superyor cells were slightly more resistant than the Δ*yor1* cells to ethidium bromide, to eosin Y and to verapamil, a modulator of mammalian P-glycoprotein ATPase activity [22] and of GSH transport by MRP1 [23]. On the other hand, overexpression of Yor1p had no effect against the toxicity of o-phenanthroline or of fluorescein. Finally, we searched for a possible involvement of Yor1p in the resistance to cadmium [14]. The MIC of the metal (30 μM $CdCl_2$ in Table 2) did not change upon Yor1p overexpression.

3.2. ATPase activity of Yor1p in membranes

In previous studies, to detect a low ATPase activity associated to Yor1p, solubilization and partial purification of this protein were required [15]. In the present study, we made every effort to minimize contaminant ATPase activities in membrane preparations. For this purpose, specific inhibitors of phosphatases and of vacuolar or mitochondrial ATPases (0.2 mM ammonium molybdate, 50 mM KNO_3 and 10 mM NaN_3, respectively) were included in the assays. In addition, membrane fractions were washed in a buffer containing 1 M NaCl and repelleted. This washing step allowed further Yor1p-enrichment by eliminating >50% of the proteins retained in the membrane extract, without loss of Yor1p as shown in Fig. 3A. SDS-PAGE analysis of membrane preparations isolated from the Yor1p-deleted strain (Superyor-Δyor) and from Superyor shows a band of approximately 170 kDa (the expected M_r of Yor1p is 166,600 Da) in the Superyor membranes, but not in the Superyor-Δyor ones (Fig. 3B). Relative Yor1p enrichment was estimated at 8–10% of total membrane proteins by densitometric scanning of the Coomassie-stained gel.

The ATPase activity was assayed at 37 °C, pH 7.5, by measuring the release of [^{32}P]-Pi from 10 μM [γ-^{32}P]ATP in the presence of 2 mM $MgCl_2$ and of several phosphatases and ATPases inhibitors, as described in Materials and methods (Fig. 3C). Membrane extracts containing overproduced Yor1p displayed an ATP hydrolytic activity of 7.2±0.3 nmol of Pi released per min and per mg membrane proteins. The activity in extracts from the Yor1p-deleted strain Superyor-Δyor was 4.8±0.2 nmol P_i min^{-1} mg^{-1}. When protein extracts were omitted, the rate of spontaneous ATP hydrolysis was less than 0.1 pmol min^{-1}. Using several preparations of Yor1p-depleted or overexpressing membranes, similar differences in activities were reproducibly observed.

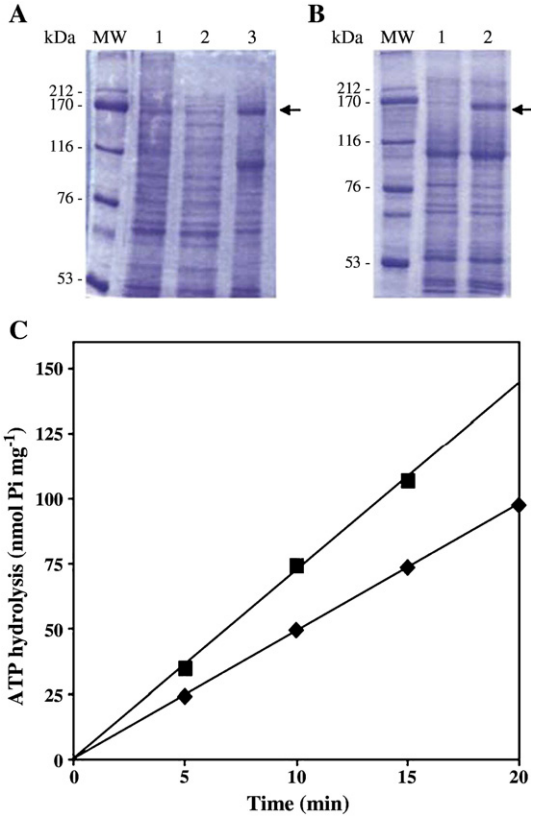

Fig. 3. (A, B) Coomassie blue-stained SDS-PAGE profiles obtained along the preparation of Superyor membranes. (A) Lane 1, Membrane pellet from strain Superyor (20 μg membrane proteins), Lane 2, Supernatant obtained after washing with a 1 M NaCl solution (12.5 μg proteins), Lane 3, Crude membrane extract (pellet obtained after the 1 M NaCl wash) from strain Superyor (7.5 μg proteins). (B) Lane 1, Crude membrane extract from strain Superyor-Δyor (10 μg proteins), Lane 2, Crude membrane extract from strain Superyor (10 μg proteins). *MW,* molecular weight marker. The position of Yor1p is indicated by an arrow. (C) Time-dependence of the ATPase activity associated to membrane proteins (3 μg) prepared from Superyor (■) and Superyor-Δyor (♦). ATP hydrolysis was measured at 37 °C in 50 mM Tris–HCl (pH 7.5) containing 10 μM [γ-^{32}P]ATP (200 Ci/mol), 2 mM $MgCl_2$, 0.2 mM $(NH_4)_6Mo_7O_{24}$, 50 mM KNO_3, 5 mM NaN_3, 10 μM EDTA, 2% glycerol and 0.2 mM DTT. Experimental error is estimated to be ±10% for each data point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To establish that the above difference between the ATPase activities in Superyor and Superyor-Δyor membranes was specific of Yor1p, we searched for mutations capable of abolishing ATP hydrolysis by this protein. Structural analyses of bacterial ABC-transporters MJ0796 [24], HisP [25], and GlcV [26] have indicated that the conserved Glu residue immediately adjacent to the conserved "Walker B" Asp residue might play the role of a catalytic base during ATP hydrolysis. In agreement with this idea, mutagenesis of this residue to a Gln in either the NBD1 or the NBD2 of P-gp results in strongly impaired drug transport and ATPase activities [27,28]. Similar effects are obtained upon mutation of this Glu in the NBD2 of human MRP1 [29] or in that of the *Bacillus subtilis* multidrug transporter BmrA [30]. In the bacterial histidine permease, another conserved residue, the histidine belonging to the H-loop, is essential for hydrolysis, while ATP binding is not impaired [31]. Mutations in the maltose transporter [32], in the HylB transporter [33] or in MRP1-NBD2 [34], confirmed that this conserved His is essential for activity. Therefore, we substituted the putative catalytic Glu 1392 residue of Yor1p with a non-acidic Gln one to create the Yor1p-E1392Q mutant. Another mutant having an arginine residue substituted for His 1423 was called Yor1p-H1423R. Both mutants were produced in a previously constructed histidine-tagged derivative of *YOR1* (for details on the construction of *YOR1-(His)$_{10}$* see Materials and methods). SDS-PAGE analysis of membrane preparations (Fig. 4A) showed that the levels of expression of both mutant proteins are similar to those of native or of His-tagged Yor1p. As expected, the resistance of both mutants strains towards oligomycin was extremely diminished, as compared to that of Superyor, thus suggesting that translocation of this compound had become severely impaired (Fig. 4B). On the other hand, resistance of cells expressing the intact Yor1p-$(His)_{10}$ derivative was identical to that of the Superyor cells.

The ATPase activity of Yor1p-$(His)_{10}$ expressing membranes (6.8±0.5 nmol P_i min^{-1} mg^{-1}) was very close to that measured in Superyor membrane preparations (7.2±0.3). Upon introduction of either the E1392Q or the H1423R mutation, the ATPase activity of Yor1p-$(His)_{10}$ membranes was reduced (4.9±0.2,

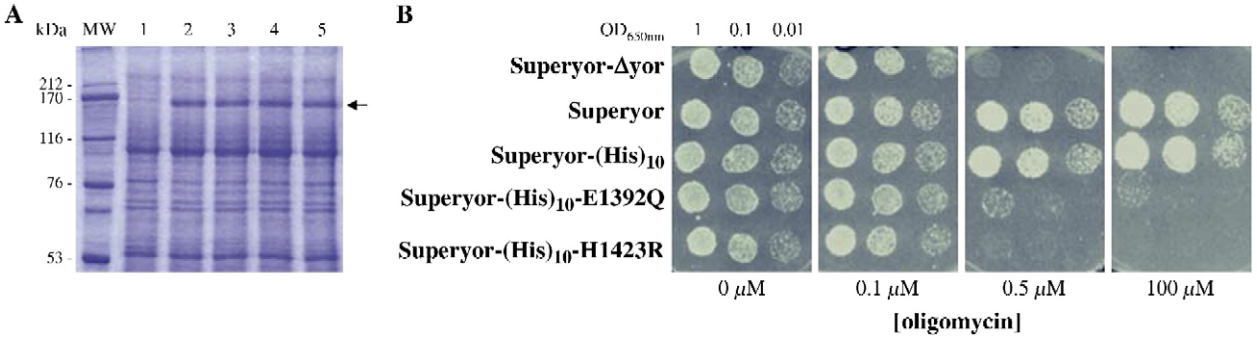

Fig. 4. Expression (A) and drug resistance (B) of the different Yor1p constructs. (A) Coomassie blue-stained SDS-PAGE profiles. Lane 1, membrane extract from strain Superyor-Δyor, Lane 2, membrane extract from strain Superyor, Lane 3, membrane extract from strain Superyor-$(His)_{10}$, Lane 4, membrane extract from strain Superyor-$(His)_{10}$-E1392Q, Lane 5, membrane extract from strain Superyor-$(His)_{10}$-H1423R. 10 μg of membrane proteins were applied in each well. *MW,* molecular weight marker. The position of Yor1p is indicated by an arrow. (B) Effect of oligomycin on the growth of *S. cerevisiae* strains Superyor-Δyor, Superyor, Superyor-$(His)_{10}$, Superyor-$(His)_{10}$-E1392Q and Superyor-$(His)_{10}$-H1423R. Legend is as in Fig. 1A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5.0±0.2, respectively) to that of membrane extracts from the Yor1p-deleted strain (4.8±0.2). These experiments were reproduced 3 times. We therefore conclude that the difference in activity between Superyor and Superyor-Δyor extracts was small (2.4) but specific of Yor1p. Taking into account that Yor1p represents ~10% of total membrane proteins, the activity value specific of Yor1p, measured in the presence of 10 μM ATP, is around 25 nmol of released P_i min^{-1} mg^{-1}.

Rates of ATP hydrolysis as a function of ATP concentration were followed in Superyor and in Superyor-Δyor extracts. In the two cases, ATPase activity increased with the substrate concentration. After subtraction of the activity measured with Superyor-Δyor, the activity of the Superyor extracts could be fitted to the Michaelis–Menten equation. A V_{max} value of 290±10 nmol P_i min^{-1} mg^{-1} of Yor1p (0.8 s^{-1}) and a K_m value for ATP of 50±8 μM were determined.

3.3. Effect of ATPase inhibitors

In the following experiments, ATP was kept at a concentration of 10 μM in the assay. Actually, because the K_m value is 50 μM, the rate of ATP hydrolysis specific of Yor1p in Superyor extracts could have been increased by raising the substrate concentration above 10 μM. However, we observed that the rate of ATP hydrolysis in the control Superyor-Δyor extracts also increased with the ATP concentration. Such a behaviour reflects saturation, by the substrate, of the various ATPases present in the Δyor extracts. As a consequence, the ratio between the activities in the Superyor and Superyor-Δyor extracts slightly decreased when the ATP concentration was raised. This decrease was at the expense of the accuracy of the assay. Thus, to optimize accuracy while being under steady-state conditions, we chose an ATP concentration below the K_m value.

Several ATPase inhibitors were screened for their effect on the activity of both Superyor and Superyor-Δyor extracts (Table 3). Thiol reagents, such as p-chloromercuryphenyl sulfonate (200 μM) or N-ethylmaleimide (up to 2 mM), slightly inhibited Δyor or Superyor membrane ATPase activities. Ouabain (1 mM), an Na^+, K^+-ATPase inhibitor, di-cyclohexylcarbodiimide (100 μM), an F-ATPase inhibitor, and heparin (1 mg/ml), a kinase inhibitor, had no effect on either activities. The effect of vanadate, a specific inhibitor of P-ATPases and ABC-transporters, is shown in Fig. 5. The activity measured in Superyor-Δyor membranes was sensitive to the presence of this inhibitor. At 200 μM vanadate, 60% of the ATPase activity was lost. With Yor1p overexpressing membranes, the ATPase activity was also strongly inhibited by vanadate. After subtraction of the Superyor-Δyor values (Fig. 5, open circles), the ATPase activity specific of Yor1p is shown to be inhibited by more than 90% upon saturation by vanadate, with an IC_{50} around 20 μM.

Table 3
Effects of various compounds on the activity of Superyor and Superyor-Δyor membranes

	ATPase activity (nmol P_i min^{-1} mg^{-1})	
	Superyor-ΔYor	Superyor
Control	4.8±0.2	7.2±0.3
pCMPS, 200 μM	4.2±0.2	6.1±0.1
NEM, 2 mM	3.7±0.3	5.6±0.2
Ouabain, 1 mM	4.6±0.1	7.1±0.3
DCC, 100 μM	4.7±0.2	6.6±0.2
Heparin, 10 μM	4.5±0.1	6.4±0.2
Vanadate, 200 μM	1.9±0.2	2.2±0.3

The ATPase activity of 3 μg membrane proteins was followed for 15 min in the presence of 2 mM $MgCl_2$, as described in Materials and methods. The compounds under study were added in the assays at the concentrations indicated in the table. Data correspond to the mean±SD of three independent experiments.

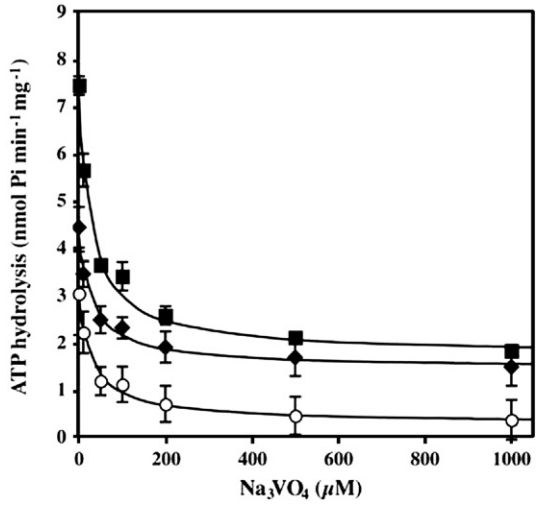

Fig. 5. Inhibition by o-vanadate of the ATPase activity of membrane proteins (3 μg) prepared from Superyor (■) and Superyor-Δyor (◆). The ATPase activity of 3 μg membrane proteins overexpressing Yor1p was measured for 15 min as in Fig. 3C, in the presence of increasing concentrations of o-vanadate. The curve with open circles was calculated as the difference between the values obtained using Superyor and Superyor-Δyor membrane proteins. Therefore, the curve describes the inhibition of the ATPase activity of Yor1p by vanadate. O-vanadate stock solutions were prepared as described [60] and incubated for 2 min at 95 °C prior to addition to the assays.

3.4. Effect of drugs

Substrate-stimulated ATPase activity of membranes expressing ABC-transporters has been reported in several cases. For instance, the ATPase activity of mammalian P-gp in membrane preparations is stimulated by up to 5-fold upon addition of verapamil [35,36]. In contrast, oligomycin, a potential substrate of Yor1p, inhibits the ATPase activity of partially purified Yor1p [15]. In the present study, we used membrane extracts to investigate the effects of drugs towards which cells overexpressing Yor1p exhibit increased resistance. As shown in Fig. 6, none of the assayed compounds succeeded in stimulating the ATPase activity of Yor1p. Instead, nearly 95% of the Yor1p specific ATPase activity was inhibited by oligomycin, with an IC_{50} of 6.9±1.4 μM. Nearly full inhibitions by rhodamine 6G (IC_{50}=1.2±0.2 μM), rhodamine B (IC_{50}=5.9±0.5 μM), rhodamine 101 (IC_{50}=1.1±0.2 μM), verapamil (IC_{50}=42±12 μM) and doxorubicin (IC_{50}=73±5 μM) were also observed. The ATPase activities of Superyor-Δyor membrane preparations were measured, in parallel, in the presence of the above drugs. In all cases, the assayed drug did not inhibit the activity by more than 10%.

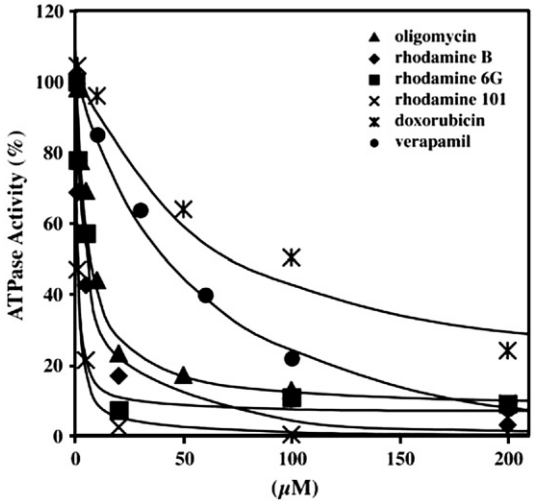

Fig. 6. Effect of potential substrates on the ATPase activity of membrane-bound Yor1p. The ATPase activity of 3 μg membrane proteins overexpressing Yor1p was measured as in Fig. 3C, in the presence of increasing concentrations of indicated drugs. Rate values with membranes prepared from the Δ*yor1* strain were measured in parallel and subtracted. Data are presented as percentages of control activity (100%) assayed in the absence of any drug. Oligomycin, rhodamine 6G and rhodamine 101 were assayed in the additional presence of 2% ethanol to ensure solubilization of these drugs.

To analyse in more details this paradoxical result, oligomycin was used as model "substrate". First, to exclude the possibility that this drug may cause denaturation of the protein, we preincubated Yor1p expressing membranes in the presence of a nearly half-inactivating concentration of oligomycin (5 μM). After addition of the drug, ATPase hydrolysis was followed over time, for a period of 2 h. No further inhibition of Yor1p hydrolytic activity was observed in these conditions. Another explanation might be that oligomycin prevents the binding of ATP to the NBDs. Indeed, disulfiram, a modulator of P-gp and of MRP1 impairs the binding of ATP [37]. Therefore, we searched for an effect of oligomycin on the binding of 8-azido-[α-^{32}P]ATP, a photoaffinity analog of ATP, which has been extensively used to probe the nucleotide binding sites of other ABC-transporters. Membrane preparations were incubated for 20 min on ice with 2 mM $MgCl_2$ and with 5 or 10 μM of the reagent. The samples were analysed by SDS-PAGE and autoradiographed. The intensity of Yor1p labelling was higher with 10 μM 8-azido-ATP than with 5 μM. We therefore concluded that, at 5 μM of the reagent, (i) the binding site for 8-azido-ATP was not saturated and (ii) the labelling reaction was not completed during the time of the assay. Next, we designed a sensitive assay containing a 5 or 1 μM fixed concentration of 8-azido-ATP and variable ATP concentrations to verify that the two nucleotides were in competition to bind Yor1p. As expected, excess ATP cancelled the reaction of the photo-reagent. The same assay, with variable oligomycin concentrations (up to 100 μM) instead of ATP, was used to search for a possible interference of oligomycin with the binding of 8-azido-ATP to Yor1p. The result was negative. Thus, inhibition by oligomycin of the ATPase activity of Yor1p cannot be explained by an interference of this drug with the ATP binding capacity of the NBDs.

4. Discussion

4.1. Substrate specificity of Yor1p

Yor1p was initially identified on the basis of its ability to elevate oligomycin resistance when it was overproduced from a high-copy-number plasmid [13]. At the same time, Cui et al. showed that Yor1p was important to confer tolerance to organic anions that contain carboxyl groups [14]. As oligomycin does not contain anionic groups, the latter authors speculated that this drug might be modified *in vivo* into a compound containing a carboxyl group or that it might be pumped out as an anionic conjugate. However, Yor1p was later implicated in the resistance to aureobasidin A, an antifungal antibiotic which structure is that of a cyclic depsipeptide [38]. In this study, we show that overexpression of Yor1p increases the cellular resistance to the P-glycoprotein modulator verapamil and to cyclic compounds that do not possess a carboxyl group, like doxorubicin or tetracycline. Moreover, we observe that the Superyor strain is more resistant than the Δ*yor1* strain to rhodamine 6G, a non-anionic analogue of rhodamine B. Therefore, like yeast Pdr5p [21] or mammalian P-glycoprotein [39], Yor1p confers resistance to a broad range of structurally unrelated molecules.

We find that overexpression of Yor1p is accompanied by increased resistance to rhodamine 6G. However, upon deletion of *YOR1*, the sensitivity of yeast cells to this drug was not changed [20]. Possibly, the expression in yeast of the products of other multidrug-resistance genes trans-dominate an effect of Yor1p on the transport of rhodamine 6G. Here, because of its large overexpression, Yor1p can be shown to be involved. Such a situation is reminiscent of the resistance to camptothecin which is principally mediated by Snq2p. Deletion of *PDR5* or of *YOR1* had little effect on camptothecin sensitivity. Yet, overexpression of Pdr5p increased the resistance of yeast to this drug [40].

In subclass II.1, Yor1p is homologous to Ycf1p which is involved in cadmium resistance [11]. Thus, an overlapping substrate specificity of the two transporters is a possibility. In agreement with this idea, earlier studies showed that Δ*yor1* cells were more sensitive to cadmium than the parental cells [14]. More recently, various Yor1p-deleted or overproducing strains were compared [41]. It was shown that, in the presence of a functional Ycf1p, deletion of Yor1p did not increase cadmium toxicity and that overexpression of Yor1p conferred only limited resistance to cadmium. Actually, growth at low temperature was required to evidence such a resistance. Thus, even though Yor1p has resemblance with Ycf1p, its role in cadmium detoxification appears reduced. Here, by growing cells in rich medium at 30 °C, we did not succeed in showing an effect of Yor1p on the resistance against cadmium.

The case of verapamil also deserves discussion. We show in this study that Yor1p confers slight but significant resistance against this alkaloid. This suggests that intact or modified verapamil is a substrate of Yor1p. In favour of this idea, the ATPase activity of the ABC-transporter in membrane preparations is inhibited with a verapamil IC_{50} of ~50 μM. Verapamil

has been reported to modulate transport of GSH by Ycf1p [42] and MRP1 [23]. In the latter case, indirect action of verapamil on the GSH status of the cells has been proposed, rather than direct competition of verapamil for the MRP1 substrate binding site. Thus, distinct modes of action of verapamil on MRP1 and Yor1p functions can be ascribed to the fact that Ycf1p, not Yor1p, is the functional homologue of MRP1 in yeast.

4.2. ATPase activity of Yor1p in membrane preparations

In this study, we characterized the ATPase activity of Yor1p in membrane enriched extracts. Detection of an ATPase activity specific for the presence of Yor1p required a step of washing at high ionic force. This procedure allowed a further two-fold enrichment of the membranes in Yor1p. Care was taken to minimize contaminant ATPase activities by including to the solutions several inhibitors specific of vacuolar and mitochondrial ATPases and of phosphatases. In these conditions, a basal ATPase activity of 0.8 s^{-1}, specific of Yor1p, could be measured. Attribution of this activity to Yor1p is supported by the fact that mutations of essential residues in NBD2 reduced the value of the rate of ATP hydrolysis by membranes overexpressing Yor1p to that by Δyor membranes. Moreover, the Yor1p ATPase activity which we report here (V_{max}=300 nmol min^{-1} mg^{-1}) is comparable to that previously measured (V_{max}=500 nmol min^{-1} mg^{-1}) with a solubilized and partially purified Yor1p sample [15]. It is difficult to compare V_{max} of different ABC proteins in membrane preparations, as assay conditions are variable and overproduction levels are not often indicated. However, the Yor1p activity given here is at least 10 times lower than that of yeast membrane-bound Pdr5p (5 μmol min^{-1} mg^{-1} of Pdr5p) measured in similar conditions [43], or than that of overproduced Chinese hamster P-glycoprotein (4–9 μmol min^{-1} mg^{-1} of P-gp) in its native membrane environment [35]. Contrasting with its low ATPase activity, Yor1p displays a K_m value for ATP of 50 μM, one order of magnitude smaller than the K_m of membrane-bound Pdr5p (0.5 mM) or of Chinese hamster P-gp (1.3 mM). Low ATPase activities coupled to high affinities for the nucleotide have been reported for purified MRP1 [44] and CFTR [45], both of which are members of the ABCC subfamily. It is noteworthy that, in contrast with many other ABC proteins, the ABCC-proteins are characterized by relatively divergent NBDs. Alignment of the primary sequence of CFTR with those of other ABC-transporters shows that several highly conserved residues in NBDs are not conserved in CFTR NBD1. Actually, the NBDs of other ABCC family members also show asymmetric sequence abnormalities, like those in CFTR. In the case of Yor1p, the conserved glutamine of the Q-loop in NBD1 is missing. Several studies report different behaviours of the ABCC's NBD1 and NBD2, in particular at the level of ATP binding and hydrolysis [46–49]. It has been suggested that, in CFTR and MRP1, NBD1 binds ATP with high affinity but sustains little hydrolysis, whereas NBD2 is hydrolytically active [50,51]. Thus, a slow release of the bound nucleotide from NBD1 could account for the rather low ATPase activity values associated to the ABCC subfamily members.

4.3. Effect of potential substrates

Substrate-stimulated ATPase activity has been observed by using plasma membrane preparations as well as proteoliposomes-reconstituted purified ABC-transporters [35,36,44,52,53]. It was, therefore, surprising to find that ATP hydrolysis by membrane-bound Yor1p is inhibited rather than stimulated by the presence of its potential substrates. Yet, the observation of an inhibition clearly indicates that these compounds interact with Yor1p. Inhibition of the ATPase activity of Pdr5p has previously been reported [54–56]. In the case of flavonoids, a partial overlap of the drug and of the nucleotide binding sites was invoked to account for the inhibition of Pdr5p activity [54]. Here, we show that, at least in the case of oligomycin, binding of the drug does not hinder that of the nucleotide. Therefore, inhibition of Yor1p ATPase activity by oligomycin does not simply result from a competition with ATP at the nucleotide binding site.

Failure to detect a drug-stimulation of the ATPase activity of an ABC-protein has already been reported. This is, for instance, the case of *Candida albicans* Cdr1p, a Pdr5p homolog, obtained upon overexpression in the same host *S. cerevisiae* strain as the one used here [57]. This is also the case of the human ABCG2 transporter expressed in insect cells. In the latter case, ATP hydrolysis could not be stimulated by any of the potential substrates that were assayed. An inhibition of the ATPase activity was even observed in the presence of Hoechst 33342, a transported substrate of ABCG2 [58]. This behaviour was explained by assuming the presence in the membrane preparations of an unknown ABCG2 substrate that would ensure maximal rate of ATP hydrolysis. Therefore, competition between this endogenous activator and any added substrate would lead either to conservation, at best, or to reduction of the rate of ATP hydrolysis. Another explanation may be that preparation of the membranes has removed an unknown molecule that is required for ATPase powered translocation. In the absence of such a co-factor, a substrate would still bind Yor1p but the basal ATPase catalytic cycle of the transporter would be blocked. For instance, uptake of vincristine by MRP1-enriched inside-out membrane vesicles is stimulated by the presence of reduced glutathione [59]. Actually, we searched whether the addition of GSH interfered with the ATPase activity of Superyor membranes but could not evidence an effect (results not shown). Finally, we note that, up to now, transport of drugs by Yor1p could be evidenced only *in vivo* [15]. Therefore, modifications of the Yor1p substrates inside the cell or co-transport with an, as yet undiscovered, molecule cannot be excluded.

To further investigate the relationship between substrate transport and ATP hydrolysis, Yor1p function will have to be analysed in defined environments. To this end, the Superyor strain was used to produce a Yor1p derivative with a C-terminal histidine-tag. As shown here, this additional sequence neither compromises the overexpression nor the hydrolytic properties

of Yor1p. Experiments are underway to solubilize tagged-Yor1p and to isolate it, while keeping intact its ATPase activity.

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