Two different dihydroorotate dehydrogenases from yeast *Saccharomyces kluyveri*

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Abstract Genes for two structurally and functionally different dihydroorotate dehydrogenases (DHODHs, EC 1.3.99.11), catalyzing the fourth step of pyrimidine biosynthesis, have been previously found in yeast *Saccharomyces kluyveri*. One is closely related to the *Schizosaccharomyces pombe* mitochondrial family 2 enzymes, which use quinones as direct and oxygen as the final electron acceptor. The other one resembles the *Saccharomyces cerevisiae* cytosolic family 1A fumarate-utilizing DHODH. The DHODHs from *S. kluyveri*, *Sch. pombe* and *S. cerevisiae*, were expressed in *Escherichia coli* and compared for their biochemical properties and interaction with inhibitors. Benzoates as pyrimidine ring analogs were shown to be selective inhibitors of cytosolic DHODs. This unique property of *Saccharomyces* DHODHs could appoint DHODH as a species-specific target for novel anti-fungal therapeutics.

Keywords: *Saccharomyces kluyveri*; *Schizosaccharomyces pombe*; *Saccharomyces cerevisiae*; Dihydroorotate dehydrogenase; Protein expression; Inhibition

1. Introduction

In all organisms the fourth step of pyrimidine de novo synthesis is catalyzed by the flavoenzyme dihydroorotate dehydrogenase (DHODH, EC 1.3.99.11). Based on subcellular localization studies, electron acceptors and sequence similarities, the known DHODH proteins are divided into two families [1]. The family 1, which includes soluble proteins of milk fermenting Gram-positive bacteria, Archea and a few unicellular eukaryotes, is further divided into two subgroups, family 1A and family 1B (Table 1, [1–10]). The DHODH proteins from family 1A are homodimeric, those from family 1B are heterodimeric. The family 2 DHODHs are monomeric, either attached to membranes as in Gram-negative bacteria related to *Escherichia coli* or located as integral membrane proteins facing the outer side of the inner mitochondrial membrane as in mammals, insects, plants, and some unicellular organisms (Table 1, [1,4,6,11–17]). In these organisms, DHODH functionally links the pyrimidine biosynthetic pathway via quinones to the respiratory chain and thus to molecular oxygen.

A majority of the facultative fermentative yeasts requires small amounts of oxygen for growth even through alcohol fermentation [18]. *Saccharomyces cerevisiae* contains a cytosolic DHODH, and is able, if supplemented with certain compounds, to grow under strictly anaerobic conditions [19]. In contrast, *Schizosaccharomyces pombe* as a microaerophilic yeast absolutely requires oxygen for growth. This is in part related to a mitochondrially bound DHODH [4]. Some prokaryotic organisms, such as *Lactococcus lactis* [1,2] and *Enterococcus faecalis* [3] contain two cytosolic DHODHs, one of family 1A and one of family 1B (Table 1). Although the specific individual roles of these DHODHs are not precisely known, it has been suggested that one of them may participate in the catabolic reaction of orotate reduction [19].

The importance of sufficient pyrimidine nucleotide pools for cell growth, metabolism and multiplication has determined the biosynthetic enzymes as targets for the development of antiproliferative agents. In view of the enormous structural differences between DHODH from various organisms, pharmacological inhibitors have been already applied to reduce aberrant immunological reactions [20,21], to interfere in the multiplication of animal parasites and parasitic protozoa [22,23] and to support antiviral therapies [24]. *Candida albicans* is still the major fungal agent of human disease, but other yeasts, including *S. cerevisiae* isolates, are recently of growing concern [25]. New strategies and agents, e.g., the identification of DHODH as an anti-fungal target, are in the developmental stage [26–29].

Another yeast, *Saccharomyces kluyveri*, has recently been intensively studied for metabolism of nucleic acid precursors. In contrast to *S. cerevisiae*, both the pyrimidine and purine catabolic pathways are present and are functional in *S. kluyveri* [30]. Through our attempts to compare the de novo pyrimidine biosynthesis between these two yeasts and in search for yeast-specific anti-pyrimidines, we have previously cloned two DHODHs from *S. kluyveri* [6] (Table 1). From sequence analysis and assignment to DHODH family 1 and family 2, the different subcellular location in this eukaryotic cell became evident. This yeast species is apparently able to synthesize

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Abbreviations: DHO, dihydroorotate; DHODH, dihydroorotate dehydrogenase (EC 1.3.99.11); Q_D, decylubiquinone; DCIP, 2,6-dichlorophenol-indophenol; 3,4-DHB, 3,4-dihydroxybenzoic acid; 3,5-DHB, 3,5-dihydroxybenzoic acid

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Table 1
Protein families of DHODH

Family	Organism and references	Subcellular localization	Electron acceptors
1 A	Gram-positive bacteria *L. lactis* [1,2] *E. faecalis* [3]	Cytosolic	Fumarate
	Unicellular organisms *S. cerevisiae* [4,5] *S. kluyveri* [6]	Cytosolic	Fumarate
	Trypanosamatids [7,8]	Cytosolic	Fumarate, ?[a]
1 B	Gram-positive bacteria *L. lactis* [1,2] *E. faecalis* [3] *Bacillus subtilis* [9] *Clostridium oroticum* [10]	Cytosolic	NAD
2	Gram-negative bacteria *E. coli* [1,11]	Membrane bound	Quinone
	Unicellular organisms *Sch. pombe* [4] *S. kluyveri* [6] *Toxoplasma gondii* [12] *Plasmodium falciparum* [13]	Membrane bound	Quinone
	Plants [14], insects [15], mammals [16,17]	Membrane bound	Quinone

Examples from various organisms.
[a] DHODH from theses organisms was also shown to use molecular oxygen as electron acceptor.

pyrimidines for cell growth and proliferation under aerobic or anaerobic conditions. A similar capability has been previously created by transformation of otherwise strictly aerobic *Pichia stipidis* with the *S. cerevisiae URA1* gene [31].

The present biochemical characterization of the two recombinant *S. kluyveri* enzymes (Accession Nos. AF 452108 and AF 452109 in the GenBank data base) in comparison to the recombinant DHODH from *S. cerevisiae* and *Sch. pombe* was performed to better describe the functional role of yeast DHODH and to define their potential value as targets for inhibition of the pyrimidine biosynthesis in fungi.

2. Materials and methods

2.1. Materials

Special chemicals L-dihydroorotic acid, decylubiquinone (Q_D), 2,6-dichlorophenol-indophenol (DCIP), and fumarate were from Sigma, Germany. Anhydrotetracycline was from Acros, Belgium. The inhibitors studied were A77-1726, 2-hydroxyethylidene-cyanoacetic acid 4-trifluoromethyl anilide, the active metabolite of ARAVA™ (HMR Deutschland GmbH – Aventis); brequinar sodium (NSC 368390, 6-fluoro-2-(2'-fluoro-1,1'-biphenyl-4yl)-3-methyl-4-quinoline carboxylic acid (DuPont Pharma GmbH); atovaquone, trans-2-(4-(chlorophenyl) cyclohexyl)-3-hydroxy-1,4-naphthoquinone, 566C80 (The Wellcome Foundation, Dartford, Kent); 3,4-dihydroxybenzoic acid (3,4-DHB) and 3,5-dihydroxybenzoic acid (3,5-DHB, Sigma).

Synthetic oligonucleotides were from DNA Technology A/S, Aarhus, Denmark:

ura1for: 5′CTAGTCTAGATAACGAGGGCAAAAAATGACAGCCAGTTTAACTACCAAG3′;
ura1rev: 5′CCGCAATTGAATGCTGTTCAACTTCCCACG3′;
SpDHODHfor: 5′CTAGTCTAGATAACGAGGGCAAAAAATGTATCAACGATCTTTGTTTCGCG3′;
SpDHODHrev: 5′CCGGAATTCTTCTTCCTTGCCAATTATGTC3′;
cSkDHODH-for: 5′CTAGTCTAGATAACGAGGGCAAAAAATGTCTGCATCCTTAGCTATCAACT 3′;
cSkDHODH-rev: 5′CCGCAATTGCAGACTGTTTAACTTACCACGAAATTGCTCGATCGATGTGT3′;
SkDHODHfor: 5′CTAGTCTAGATAACGAGGGCAAAAAATGCATTCAAGACCTCTACCAACAT3′;
SkDHODHrev: 5′TCCCCCGGGTTTACTGT TTCTCCGATGATTTG3′;
ZG-SkURA1Sc5′: 5′CTG CAT CCT TAG CTA TCA3′;
ZG-SkURA1Sc3′: 5′TCT TAG CCA TTC CCA TGC3′.

2.2. Construction of expression vectors

Cytosolic DHODH from *S. cerevisiae*: the ORF of the enzyme was amplified from the plasmid pFL39 containing the URA1 gene [32] with the primers ura1for and ura1rev. The resulting PCR fragment was cut with *Xba*I/*Mfe*I and subsequently ligated into P343 pre-cut with *Xba*I/*Eco*RI [30,6]. The resulting plasmid was named P609 and the recombinant enzyme is referred to as SCcyt-DHODH. Mitochondrial DHODH from *Sch. pombe*: the ORF of the enzyme was amplified from the plasmid pFLREC1 [4] with the primers SpDHODHfor and SpDHODHrev. The resulting PCR fragment was cut with *Xba*I/*Eco*RI and subsequently ligated into P343, pre-cut with *Xba*I/*Eco*RI. The resulting vector was named P610 and the recombinant enzyme is referred to as SPmt-DHODH. Cytosolic DHODH from *S. kluyveri*, genomic DNA from Y057 (NRRL Y-12651) was amplified by PCR with the primers ZG-SkURA1Sc5' and ZG-SkURA1Sc3'. The ORF of the enzyme was amplified from this genomic fragment with the primers cSkDHODH-for and cSkDHODH-rev. The resulting PCR fragment was cut with *Xba*I/*Mfe*I and subsequently ligated into P343, pre-cut with *Xba*I/*Eco*RI. The resulting vector was named P628 and the recombinant enzyme is referred to as SKcyt-DHODH.

Mitochondrial DHODH from *S. kluyveri*: the ORF of the enzyme was amplified from plasmid P629 [6] with the primers SkDHODHfor and SkDHODHrev. The resulting PCR fragment was cut with *Xba*I/*Sma*I and subsequently ligated into P343, pre-cut with *Xba*I/*Sma*I. The resulting vector was named P611 and the recombinant enzyme is called SKmt-DHODH. The vector constructed to express the N-terminal truncated SKmt-DHODH lacking the first 52 amino acids was named P612; and the recombinant enzyme is referred to as ΔSKmt-DHODH. All recombinant enzymes have the $8 \times$ His-tag at the C-terminus originating from the vector P343 [33,34].

2.3. Expression and purification of recombinant S. kluyveri, S. cerevisiae, Sch. pombe DHODH

Transformed *E. coli* BL21-RP (Stratagene) strains were grown to $A_{600\,nm} = 0.6$–0.7 in Luria Broth medium with 100 μg/mL ampicillin. Protein expression was induced by 200 μg/L anhydrotetracyclin and

carried out at room temperature for 24 h. The recombinant histidine-tagged proteins were gained from separation on Ni^{2+}–nitrilotriacetate agarose [34]. SKmt-DHODH and SPmt-DHODH were purified in buffer containing 20 mM sodium phosphate, 500 mM NaCl, and 0.1% Triton X-100, pH 7.4, while ΔSKmt-DHODH, SCcyt-DHODH and SKcyt-DHODH did not require the presence of detergents but 10% glycerol for purification as reported for other truncated DHODH species [35]. Protein content was determined using the Bradford protein assay with bovine serum albumin as standard. Fractions from the purification procedure were analyzed by SDS–PAGE and flavin analysis was performed spectrophotometrically as described previously [15,35]. For Western blots the proteins were handled as described [15], using ImmobiloneP from Millipore, the enhanced chemical luminescence (ECL) detection kit from Amersham, and the nitrilotriacetate-coupled horse-radish peroxidase from Qiagen, Germany.

2.4. Enzyme assays

Activity studies of the mitochondrial DHODH were performed at 30 °C in 50 mM Tris/HCl, 150 mM KCl, and 0.1% Triton X-100, pH 8. The oxidation of the substrate DHO with quinone as electron acceptor was coupled with the reduction of the chromogen DCIP (600 nm, $\varepsilon = 18\,800$ L M^{-1} cm^{-1}). The detergent was omitted when cytosolic DHODHs were assayed without the quinone but with DCIP as artificial electron acceptor only [6,35]. The reduction of fumarate, which was suggested to be the natural acceptor of the cytosolic enzymes [1,6], was followed by monitoring the increase in orotate absorbance at 280 nm ($\varepsilon = 7500$ L M^{-1} cm^{-1}) in detergent-free buffer. In the presence of quinones, the orotate determination was done at the isosbestic wavelength of Q_D (300 nm, $\varepsilon = 2950$ L M^{-1} cm^{-1}). The assay buffer without Triton X-100 was used for 30 mM stock solutions of 3,4-DHB, 3,5-DHB, A771726 and brequinar sodium. 2.5 mM atovaquone was solubilized in dimethylsulfoxide. The effect of these compounds on yeast DHODH activity was followed using the standard chromogen reduction assay.

2.5. Kinetic analysis

In studying the variation of the initial velocity over a range of substrate concentrations, the concentration of all enzymes in the assays was far below that of the substrates (approximately 10^{-7}). Kinetic data were evaluated by non-linear regression analysis using the Michaelis–Menten equation $v = V_{max} \times [S]/(K_m + [S])$ as described previously [34,35]. To determine the type of inhibition by the dihydroxybenzoates, V_{max} and K_m values were determined at three different inhibitor concentrations [36]. Their deviations were considered to determine whether the inhibition was competitive, uncompetitive or non-competitive. Once the inhibition pattern was determined, the appropriate equation for competitive inhibition $v = V_{max} \times [S]/\{K_m \times (1 + [I]/K_{ic}) + [S]\}$, non-competitive inhibition $v = V_{max} \times [S]/\{K_m \times (1 + [I]/K_{ic}) + (1 + [I]/K_{iu}) \times [S]\}$ or uncompetitive inhibition $v = V_{max} \times [S]/\{K_m + (1 + [I]/K_{iu}) \times [S]\}$ was fit to the entire data set. K_{ic} is the competitive inhibition constant, K_{iu} is the uncompetitive inhibition constant [36].

3. Results and discussion

3.1. Expression of recombinant S. kluyveri, S. cerevisiae, Sch. pombe DHODH

Expression vectors were constructed to produce full-length mitochondrial *S. kluyveri* DHODH (SKmt-DHODH), mitochondrial *Sch. pombe* DHODH (SPmt-DHODH), cytoplasmic *S. kluyveri* DHODH (SKcyt-DHODH), cytoplasmatic *S. cerevisiae* DHODH (SCcyt-DHODH), and a N-terminal truncated mutant of the mitochondrial *S. klyuveri* protein (ΔSKmt-DHODH), lacking the putative bipartite mitochondrial targeting motif. Such a typical amino-terminal sequence was identified to promote import (targeting sequence) and correct insertion (transmembrane sequence) of the animal DHODH into the mitochondrial membrane [15–17].

It was shown that similar mutants of DHODH from different organisms could be gained and handled without detergents for characterization and routine inhibition studies [35]. Also, the protein crystallization of human and rat DHODH performed

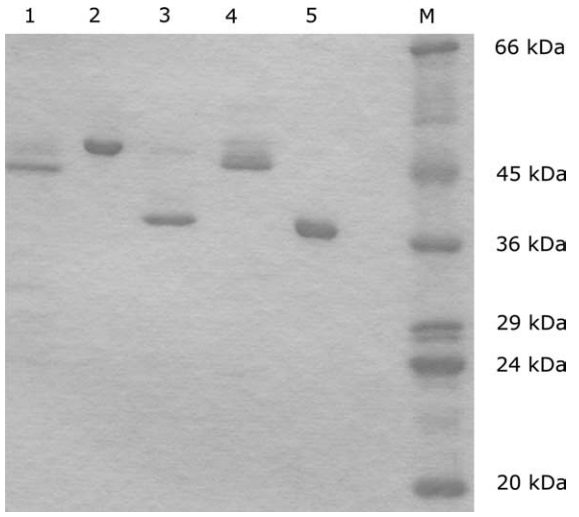

Fig. 1. SDS–PAGE of the recombinant yeast DHODH. All DHODH proteins were expressed as C-terminal 8 × His-tagged proteins and purified on Ni^{+}-nitrilotriacetate chromatography. M: Molecular mass marker. Lane 1, SKmt-DHODH; lane 2, ΔSKmt-DHODH; Lane 3: SKcyt-DHODH; Lane 4: SPmt-DHODH; Lane 5: SCcyt-DHODH; (2 μg protein per lane).

to date utilized N-terminal truncated histidine-tagged fragments [37,38]. Following the purification of the full-length and truncated yeast DHODH by metal-chelate affinity chromatography, the SDS–PAGE in Fig. 1 shows that protein bands of all enzymes were of the expected molecular mass range, above and below the 45 kDa marker protein. However, the band of the shortened protein ΔSKmt-DHODH (Fig. 1, lane 2) is above those of SKmt-DHODH and SPmt-DHODH, respectively (Fig. 1, lanes 1 and 4). This unconventional behavior can be explained, because the long N-terminal mitochondrial targeting sequences of the full-size proteins contain numerous positively charged amino acids and consequently could bind a higher amount of SDS molecules. This elevated binding would cause a relatively higher negative charge of the corresponding proteins. The Western blot in Fig. 2, performed to detect specifically the histidine-tagged proteins, shows the respective position of the different DHODH species. In Fig. 2 on lanes 9 and 10, the strong bands above the 36 kDa marker protein are in agreement with the value of 37.1 kDa calculated from the histidine-tagged *S.cerevisiae* protein. The weak bands above the 66 kDa marker could indicate an aggregated form of the *S. cerevisiae* DHODH, although dimeric proteins are not expected in the presence of reducing agent. The purified recombinant SCcyt-DHODH was found to be a dimeric protein when analyzed on Bio-Prep SE-1000/17 (BioRad) chromatography (data not shown). A homodimer of 72 kDa was obtained from sucrose-density-gradient centrifugation of native DHODH isolated from *S. cerevisiae* [39]. Dimerization of the cytosolic family 1A DHODH protein seems to be a characteristic feature to obtain structure stabilization, whereas the stability is provided by insertion in or attachment to the membrane in the case of the family 2 DHODHs [11,15,17]. The yield of recombinant proteins purified from 1 L of *E. coli* cultures was considerably different for the cytosolic and mitochondrial forms of yeast DHODH when cultured under fairly identical conditions: 20 mg SCcyt-DHODH and 15 mg SKcyt-DHODH versus 2.2 mg SPmt-DHODH, 1.5 mg SKmt-DHODH. An increase in the protein yield of the *S. klyuveri* enzyme was obtained from

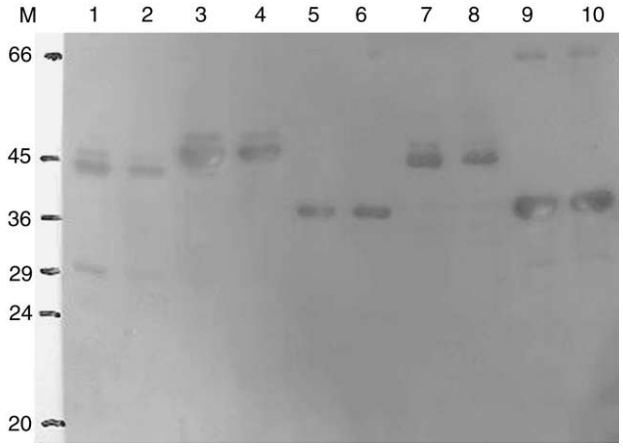

Fig. 2. Western blot of recombinant yeast DHODH. Samples were separated by SDS–PAGE, electrotransferred on ImmobiloneP, and analyzed by Western blotting using nitrilotriacetate-coupled horseradish peroxidase for the detection of the histidine-tagged proteins in combination with the ECL detection kit. M: molecular mass (kDa). Lane 1, 1 μg SKmt-DHODH; lane 2, 0.2 μg SKmt-DHODH; lane 3, 1 μg △SKmt-DHODH; lane 4, 0.2 μg △SKmt-DHODH; lane 5, 1 μg SKcyt-DHODH; lane 6, 0.2 μg SKcyt-DHODH; lane 7, 1 μg SPmt-DHODH; lane 8, 0.2 μg SPmt-DHODH; lane 9, 1 μg SCcyt-DHODH; lane 10, 0.2 μg SCcyt-DHODH.

the truncated construct, 4.2 mg ΔSKmt-DHODH. The flavin/protein ratio (mol/mol) as estimated from UV–Vis analysis varied: 0.05–0.1 (SCcyt-DHODH); 0.2–0.3 (SKcyt-DHODH); 0.6–0.9 (SPmt-DHODH), 0.15–0.2 (SKmt-DHODH); 0.4–0.6 (ΔSKmt-DHODH).

3.2. *Kinetic characterization of the recombinant* S. kluyveri, S. cerevisiae, Sch. pombe *DHODH*

The specific activity of the mitochondrial yeast enzymes using Q_D and DCIP as acceptors (Table 2) was in the range of 1–8 U/mg. This is considerably lower than that obtained with other recombinant DHODH preparations, which were determined using the same assay at 30 °C (84–125 U/mg for the rat and 100–150 U/mg for the human enzyme) [34,35]. Whereas a higher flavin content could underline the elevated activity of the truncated (ΔSKmt-DHODH) over the non-truncated enzyme from *S. klyuveri* (Table 2), the lowest activity was observed with the *Sch. pombe* DHODH despite a molar flavin/protein ratio of 0.6–0.9. In comparison to the rat and human species (range of K_m = 6–15 μM for DHO and K_m = 9–14 μM for Q_D, [34,35]), the K_m values for DHO and Q_D (range from 50 to 260 μM, Table 2) of the mitochondrial yeast enzymes seem to indicate an apparently lower efficiency of the yeast enzymes over their counterpart in mammals. Since the concentration of intermediates in the pyrimidine biosynthetic pathway in the yeast cell is not known, a significance of this finding for the cell growth could not be addressed.

The K_m values for DHO and the acceptor fumarate given in Table 2 with the recombinant cytosolic DHODH correlate quite well with values reported for the purified native *S. cerevisiae* enzyme, 8 μM for DHO and 45 μM for fumarate [39]. The K_m values of 100 μM for DHO and 100 μM for fumarate were evaluated with the enzyme in yeast extracts [4]. On the basis of 5–10% FMN in the recombinant SCcyt-DHODH protein, the k_{cat} values (Table 2) are comparable to those of 25 s^{-1} (DHO varied) and 23 s^{-1} (fumarate varied) reported for the native DHODH [39]. The cytosolic DHODH from *S. kluyveri* is very similar in its kinetic properties to that of *S. cerevisiae* (Table 2).

3.3. *Inhibition of the recombinant* S. kluyveri, S. cerevisiae, Sch. pombe *DHODH*

Specific inhibitors for yeast DHODH have not been described yet. Therefore, we studied the recombinant enzymes from *S. kluyveri*, *S. cerevisiae*, and *Sch. pombe* for their susceptibility to various compounds, which have already been proven as potent inhibitors of DHODHs from other species. The cinchoninic derivative brequinar was originally developed as cytostatic agent, the malononitrilamide A77-1726 has an anti-inflammatory efficacy profile and the naphthoquinone atovaquone received clinical approval to combat *Plasmodium falciparum* and *Pneumocystis carinii*.

From Table 3, it can be seen that brequinar and A77-1726 had to be applied in the millimolar range in order to cause a 10–20% reduction of activity with the cytosolic DHODH from *S. kluyveri*, *S. cerevisiae* and a 20–70% decrease with the mitochondrial enzymes from *S. kluyveri* and *Sch. pombe*. Interestingly, 250 μM of the naphthoquinone atovaquone already caused about 50% inhibition of the cytosolic DHODH and the N-terminal truncated mitochondrial mutant from *S. kluyveri* although its naphthoquinone ring mimics the ubiquinone and

Table 2
Kinetic constants of the purified full-length and truncated yeast DHODH

Substrates	DHODH	K_m (μM)	V_{max} (U/mg)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
DHO variable + Q_D + DCIP	SKmt	49 ± 7	3 ± 0.13	2.5	5.1×10^4
	ΔSKmt	16.7 ± 1.5	7.7 ± 0.2	5.7	3.4×10^5
	SPmt	257 ± 51	1.2 ± 0.08	1	3.9×10^3
DHO variable + fumarate	SKcyt	20.4 ± 2.1	4.3 ± 0.1	2.6	1.3×10^5
	SCcyt	20 ± 1.9	3.4 ± 0.8	2.1	1.1×10^5
Q_D variable + DHO	SKmt	45.3 ± 12.3	0.4 ± 0.04	0.34	7.5×10^3
	ΔSKmt	213 ± 32	4.9 ± 0.5	3.6	1.7×10^4
	SPmt	109 ± 28	2 ± 0.3	1.7	1.6×10^4
Fumarate variable + DHO	Skcyt	41.6 ± 7.4	4.5 ± 0.2	2.7	6.4×10^4
	Sccyt	115 ± 13	3.8 ± 0.1	2.3	2×10^4

The concentration of DHO was varied from 0 to 1 mM at fixed concentrations of 100 μM Q_D and 60 μM DCIP or 1 mM fumarate as electron acceptors. The concentrations of Q_D and fumarate were varied from 0 to 0.2 mM and 0 to 1 mM, respectively, at a fixed DHO concentration of 1 mM. All measurements were done in triplicate. For K_m and V_{max}, the best fit (±asymptotic S.E.M.) of the Michaelis–Menten equation to all data is given. The k_{cat} values were calculated using the equation $V_{max} = k_{cat} \times [E]$, where [E] is the total enzyme concentration and is based on one active site/monomer. U is the enzyme activity as μmol substrate/min.

Table 3
Activity of recombinant yeast DHODH in the presence of putative inhibitors

DHO (mM)	Inhibitor	SCcyt-DHODH	SKcyt-DHODH	ΔSKmt-DHODH	SKmt-DHODH	SPmt-DHODH
1	3 mM 3,4-DHB	77	59	84	92	87
0.1	3 mM 3,4-DHB	42	13	72	87	n.d.
0.01	3 mM 3,4-DHB	6	5	99	63	n.d.
1	3 mM 3,5-DHB	67*	44	95	68	100*
0.1	3 mM 3,5-DHB	24	12	68	85	n.d.
0.01	3 mM 3,5-DHB	9	12	100	81	n.d.
1	3 mM A77-1726	85	91	66	82	81
1	3 mM brequinar	81	85	59	76	32
1	0.25 mM atovaquone	51	50	55	66	89*

Relative velocities determined in chromogen reduction assays with 1 mM dihydroorotate and 0.1 mM DCIP as final electron acceptor are given. The reaction velocity of each enzyme without inhibitor was set as 100%. Values are means of three determinations which varied 2–15% of the mean; (*), 20–30% of the mean, respectively.
n.d., not determined.

Table 4
Mechanism of inhibition by hydroxybenzoates of recombinant cytosolic *S. cerevisiae* and *S. kluyveri* DHODH

Inhibitor	SCcyt-DHOD K_{ic} (μM)	SKcyt-DHOD K_{ic} (μM)	Type of inhibiton
3,4-DHB	381 ± 110	98 ± 22	Competitive
3,5-DHB	327 ± 98	115 ± 42	Competitive

Inhibitor constants for competitive inhibition K_{ic} were determined by measuring the initial velocities with varying concentrations of 1–1000 μM DHO and three concentrations of 3,4-DHB (0; 0.5; 3.0; 6.0 mM) and 3,5 DHB (0; 0.5; 1.5; 3.0 mM). The constants (±asymptotic S.E.M) are derived from best fits to all data.

exhibited a competitive inhibition pattern with the mitochondrial DHODH from mammals [36]. The benzoates 3,4-DHB and 3,5-DHB were shown only recently to inhibit the family 1A DHODH from *Lactococcus lactis* in a competitive mode of action, with 32 μM dihydroorotate and 1 mM of these compounds [40]. Here, at high concentrations only 3,5-DHB was able to depress the enzyme activity of Skcyt-DHOD to less than 50%. By lowering the DHO concentration in the enzyme assays both, 3,4-DHB and 3,5-DHB, inhibited the cytosolic DHODH of *S. kluyveri* and *S. cerevisiae* by more than 90% (Table 3), whereas the same variation in DHO concentrations did not cause a significant decrease in activity of the mitochondrial DHODH. The two dihydroxybenzoates did not suppress the activity of recombinant human DHODH at a concentration up to 2 mM (unpublished data). This implies that the two benzoates and pyrimidine-ring analogs are selective inhibitors of family 1A DHODH. From kinetic analysis (Tables 3 and 4), 3,4-DHB and 3,5-DHB were evaluated as competitive inhibitors with K_i values in the μM range. Since these were lower with SKcyt-DHODH, it can be deduced that both benzoates mimic the DHO binding more effectively with the *S. kluyveri* enzyme than with DHODH from *S. cerevisiae*.

Concluding remarks. The existence of two different DHODHs within the same eukaryotic cell with distinct subcellular location has been proven [6]. Both DHODHs and a truncated mutant from *S. kluyveri*, as well as the enzymes from *S.cerevisiae* and *Sch. pombe*, are now available as recombinant proteins for the evaluation of drug efficacy in relation to that observed or expected with the animal and human DHODH.

The dissimilarities between family 1 and family 2 DHODH of diverse organisms, and the marked species specific efficiency of DHODH inhibitors, shown in the present study and in the previous work [14,15,27,35,36], would appoint the fourth enzyme of pyrimidine biosynthesis as a promising target for specific chemotherapeutic attack in yeast.

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