Plant dihydroorotate dehydrogenase differs significantly in substrate specificity and inhibition from the animal enzymes

Alexandra Ullrich[a], Wolfgang Knecht[b,1], Jure Piskur[b], Monika Löffler[a,*]

[a] *Institute for Physiological Chemistry, Philipps-University, Karl-von-Frisch-Str. 1, D-35033 Marburg, Germany*
[b] *BioCentrum, Technical University of Denmark, DK-2800 Lyngby, Denmark*

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Abstract The mitochondrial membrane bound dihydroorotate dehydrogenase (DHODH; EC 1.3.99.11) catalyzes the fourth step of pyrimidine biosynthesis. By the present correction of a known cDNA sequence for *Arabidopsis thaliana* DHODH we revealed the importance of the very C-terminal part for its catalytic activity and the reason why – in contrast to mammalian and insect species – the recombinant plant flavoenzyme was unaccessible to date for in vitro characterization. Structure–activity relationship studies explained that potent inhibitors of animal DHODH do not significantly affect the plant enzyme. These difference could be exploited for a novel approach to herb or pest growth control by limitation of pyrimidine nucleotide pools.

Key words: Dihydroorotate dehydrogenase; Pyrimidines; Quinones; Recombinant; *Arabidopsis thaliana*

1. Introduction

In plants the de novo pathway for the biosynthesis of uridine monophosphate has a gene and cell compartment organization scheme different from that in animals [1–4]. The first three enzymes, carbamyl phosphate synthetase, aspartate transcarbamylase, dihydroorotase, are monofunctional and the last two enzymes orotate phosphoribosyltransferase and orotidine-5′-monophosphate decarboxylase, are part of the multifunctional protein UMP-synthase (for review see [5,6]). These enzymes have been localized in chloroplasts [4]. Their animal equivalents, the CAD enzyme (catalyzing steps 1–3 inclusive) and the UMP-synthase are cytosolic [7]. Dihydroorotate dehydrogenase (DHODH) catalyzes the fourth step, the oxidation of dihydroorotate to orotate. In animals this enzyme has been shown to be a monomeric integral membrane flavoprotein, located at the outer surface of the inner mitochondrial membrane, where it transfers the electrons via flavinmononucleotide (FMN) to the co-substrate ubiquinone of the respiratory chain [7–9]. On the basis of amino acid sequence comparison, the enzymes of eukaryotes – animals, plants, unicellular organisms – together with the membrane-associated DHODH from Gram-negative bacteria, have been grouped together as family 2. Their soluble counterparts in Gram-positive bacteria and *Saccharomyces cerevisiae* belong to family 1. These are homodimeric or heterodimeric enzymes that use different electron acceptors [10,11]. Due to their anti-inflammatory, immunosuppressive, cytostatic or anti-parasitic effects, DHODH inhibitors, depressing the intracellular level of pyrimidine nucleotides, have been investigated in several organisms [12–16]. The availability of the recombinant human, mouse and rat DHODH allowed mode of action studies with such inhibitors [12,17–19]; some of these are in clinical use today, e.g. the anti-rheumatic drug leflunomide (Arava™) [12], the anti-malarial drug atovaquone (Malarone™) [15] and the anti-coccidial toltrazuril (Baycox™) [16]. The development of effective compounds against *Plasmodium falciparum* and *Pneumocystis carinii* took advantage of species specific differences between DHODH from family 2. New lead structures have been identified and proposed for further application [20,21]. In contrast to the wealth of information about DHODH in animals and microorganisms, relatively little is known about DHODH in plants. The activity of DHODH has been shown to be related with functional mitochondria from pea and tomato leaves [4,22] and a cDNA clone coding for the mitochondrial DHODH from *Arabidopsis thaliana* was reported to complement a DHODH deficiency in yeast by Minét et al. [23]. In this study a functional open reading frame (ORF) for the mitochondrial DHODH from *A. thaliana* (acc. no. AF454729) is reported and the over-expressed enzyme characterized for substrate specificity and inhibition. Our work renders a necessary contribution to close the gap in the knowledge of plant pyrimidine biosynthetic enzymes.

2. Materials and methods

2.1. Materials

Special chemicals, dihydroorotate, decylubiquinone (Q_D), decylplastoquinone (PQ_D), 2,3-dimethoxy-5-methyl-*p*-benzoquinone, and 2,6-dichlorophenol-indophenol (DCIP) were from Sigma, Germany. 2,5-Dimethyl-*p*-benzoquinone (PQ_0) was from Acros, Belgium. Ubiquinone-50 (Q_{10}) was from Kaneka, Japan. A77-1726 (2-hydroxyethylidene-cyanoacetic acid 4-trifluoromethyl anilide *N*-2-hydroxyethylpiperazine) the active metabolite of ARAVA™ was obtained from Aventis, brequinar sodium (NSC 368390, 6-fluoro-2-(2′-fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium) from Du-

*Corresponding author. Fax: (49)-6421-2865116.
E-mail addresses: ullricha@mailer.uni-marburg.de (A. Ullrich), wolfgang.knecht@astrazeneca.com (W. Knecht), jp@biocentrum.dtu.dk (J. Piskur), loeffler@mailer.uni-marburg.de (M. Löffler).

[1] Present address: Astra Zeneca R&D Mölndal, S-431 83 Mölndal, Sweden

Abbreviations: DHODH, dihydroorotate dehydrogenase (EC 1.3.99.11); DHO, L-dihydroorotate; Q_D, decylubiquinone; Q_{10}, ubiquinone-50; PQ_D, decylplastoquinone; PQ_0, 2,5-dimethyl-*p*-benzoquinone; DCIP, 2,6-dichlorophenol-indophenol

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Pont Pharma GmbH, Germany. The vector pASK75 was from Biometra, Germany.

Synthetic oligonucleotides were from MWG-Biotech (Germany). AT-for1a: 5′-CTAGTCTAGATAACGAGGGCAAAAAATGGCCGGAAGGGCTGCGACGTCGTCGGCG; AT-for2a: 5′-CTAGTCTAGATAACGAGGGCAAAAAATGAGTACTGCAGATGAAGCAACCTTCTGTGGGT; AT-rev5: 5′-CGGAATTCTCTGTGATCAGCACCAATTGCTT.

2.2. Construction of expression vectors

Two vectors to express the complete DHODH and a N-terminal truncated protein lacking the first 75 amino acids were constructed. The ORFs for both DHODHs were amplified with PCR, generating a *Xba*I-site with the forward primers (AT-for1a and AT-for2a) and a *Eco*RI-site with the reverse primer (AT-rev5). The vector pPyrD ARA [23] (from Dr. M. Minét, Centre de Génétique Moléculaire, CNRF, F-91190-Gif-sur-Yvette), containing *A. thaliana* DHODH cDNA was used as a template. The PCR fragments were cut with *Xba*I and *Eco*RI and replaced the ORF of the rat DHODH in the vector pASK-C. This vector has been described in detail by Bader et al. [24]. The resulting expression vectors were named: pASK-AT and pASK-Δ75AT; the cDNA sequences were verified by sequencing (TopLab, Munich). The recombinant proteins expressed from these vectors are referred to as AT-DHODH and Δ75AT-DHODH and both have a –Glu-Phe-(His)8 tag (8xhis) at the C-terminus.

2.3. Expression and purification of A. thaliana DHODH

Transformed *Escherichia coli* XL-1 (Stratagene) were grown to A_{578nm} = 0.5–0.6 in Luria broth medium with 100 μg/ml ampicillin. Protein expression was induced by 200 μg/l anhydrotetracyclin and carried at room temperature with the addition of 100 μM FMN for 48 h. The recombinant histidine-tagged proteins were gained from separation on Ni^{2+}-nitrilotriacetate agarose [24]. AT-DHODH was purified in buffer containing 0.1% Triton X-100, while Δ75AT-DHODH did not require the presence of detergents for purification. Protein content was determined by means of the bicinchoninic acid protein assay with bovine serum albumin as standard; fractions from the purification procedure were analyzed by SDS–PAGE; flavin analysis was performed spectrophotometrically as described previously [24].

2.4. Enzyme assay

All activity studies were performed with the oligo-histidine-tagged enzyme. The oxidation of the substrate L-dihydroorotate (DHO) with the quinone co-substrate was coupled to the reduction of the chromogen DCIP. The standard assay contained 1 mM DHO, 0.1 mM Q_D, 0.06 mM DCIP, 50 mM Tris/HCl, 150 mM KCl, pH 8.0, and 0.08% Triton X-100 (for solubilization of quinones) at 30°C. The 10 mM stocks of the agents brequinar and A77-1726 were dissolved in the same buffer. Stock solutions of Q_D and the other quinones were of 10 mM or 50 mM in ethanol. For the determination of kinetic constants, the formation of orotate was directly followed by monitoring the increase in it absorbance at the isosbestic wavelength of Q_D at 300 nm (ε_{300nm} = 2950 M^{-1} cm^{-1}).

2.5. Kinetic analysis

2.5.1. K_m and k_{cat} determination. Family 2 DHODH with two different binding sites for the two substrates followed a non-classical ping-pong mechanism [25]. To determine true K_m and V_{max} values, the concentration of DHO was varied from 5 to 500 μM at five fixed concentrations of Q_D (30, 50, 100, 200, 500 μM); respectively, the concentration of Q_D was varied from 30 to 500 μM at five fixed concentrations of DHO (5, 10, 50, 200, 500 μM). The equation for a ping-pong mechanism $v = V\times[DHO]\times[quinone]/(K_{m\,(DHO)}\times[quinone]+K_{m\,(quinone)}\times[DHO]+[DHO]\times[quinone])$ was fit to the data by means of a computer program (Sigma plot 5, Jandel Scientific) that provides an iterative non-linear least squares fit to the best rectangular hyperbola. The k_{cat} values were calculated using the equation $V_{max} = k_{cat}\times[E]$, where $[E]$ = total enzyme concentration and is based on one binding site/monomer for each of the both substrates.

2.5.2. pH dependence. Initial velocities at substrate concentrations of 1 mM DHO and 0.1 mM Q_D were measured in different buffer systems (Mes/HCl, Hepes/HCl, Tris/HCl) covering a range from 5.5 to 9.0. Overlapping pH ranges were measured in two buffer systems to exclude salt effects. The equation: $v = V/[(10^{-pH}/10^{-pK_{a1}})+(10^{-pK_{a2}}/10^{-pH})+1]$ was fit to the data.

3. Results and discussion

3.1. Sequence analysis of A. thaliana DHODH

The comparison of the DHODH cDNA of *A. thaliana* (acc. no. X62909) [23] with the recently published genomic sequence of *A. thaliana* [26] revealed a divergence in one base (Fig. 1). By resequencing of the plasmid pPyrD ARA [23] we detected an additional thymidine not included in X62909. The frameshift caused by this lack in the X62909 defined a premature stop-codon resulting in a truncated ORF (Fig. 1). The new ORF for the DHODH from *A. thaliana* has been submitted to GenBank with the acc. no. AF454729. The sequence established here matches the genomic sequence of *A. thaliana*. Our prediction of the ORF for the *A. thaliana* DHODH not only resulted in a significantly prolonged C-terminus in comparison to the previously suggested ORF (acc. no. X62909) [23] but also to the ORF (acc. No. BAB11185) predicted by computional analysis of the *A. thaliana* genome. The latter can be explained by the fact that the computer predicted stop-codon lies within an intron. Interestingly, the length of the C-terminus given by our approach is very close to that known for DHODH from different mammals (Fig. 2b). The complete amino acid sequence deduced from the plant DHODH cDNA revealed a 48.5 kDa protein and 54.9% identity and 63.6% similarity with the human DHODH. The importance of the very C-terminal part for catalytic activity of DHODH was emphasized by the study of Davis et al. [27] who showed that a his364ala mutant of a N-terminal truncated (his394 in the full-length) human DHODH was inactive. We found that the expression of a C-terminal truncated *A. thaliana* DHODH in accordance to the previously predicted ORF given in acc. no. X62909 [23] resulted in an inactive protein (data not shown) in contrast to the active enzyme containing his421 (Fig. 2b).

3.2. Production of recombinant A. thaliana DHODH for evaluation of enzyme properties

Expression vectors were constructed to produce a full-length (AT-DHODH) and a N-terminal truncated (Δ75AT-DHODH) version of the *A. thaliana* protein (Fig. 2a), lacking the bipartite mitochondrial targeting motif which governs import (targeting sequence) and correct insertion (transmembrane sequence) into the mitochondrial inner membrane [9]. The arrow in Fig. 2a marks the start of the N-terminal truncated protein expressed and purified in this study. Crude bacterial extracts showed an enzyme activity of around 0.1 U/mg cell protein for AT-DHODH; the activity of Δ75AT-DHODH was around 5.0 U/mg. The different expression levels of the proteins were consistently obtained in multiple experiments. For purification purposes, the full-length protein was handled with 0.1% Triton X-100, while the truncated mutant was stable without detergents in the buffer. Following purification of the full-length and truncated DHODH, the SDS–PAGE in Fig. 3 shows protein bands in the expected molecular mass range (close to the 45 kDa marker protein). The band of AT-DHODH is below that of Δ75AT-DHODH, which is in contrast to the calculated molecular mass (AT-DHODH-8His = 50.9 kDa, Δ75AT-DHODH-8His = 43.2 kDa). This unconventional behavior can be explained, because the long N-terminal mitochondrial targeting sequence of AT-DHODH contains numerous positively charged amino acids and, consequently, could bind a higher amount of SDS molecules.

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AF454729: 1325 gagctggagctactcttgttcagctgtacacgggatttgcctatggaggacctgcccca 1384
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
X62909:   1325 gagctggagctactcttgttcagctgtacacgggatttgcctatggaggacctgccc-ca 1383

AF454729: 1385 tcccacaaataaaggaggaactggtgaaatgcttagaaagggatggcttcaagtcgatcc 1444
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
X62909:   1384 tcccacaaataaaggaggaactggtgaaatgcttagaaagggatggcttcaagtcgatcc 1443
               premature stop-codon
                                  correct stop-codon
AF454729: 1445 atgaagcaattggtgctgatcacagatgataaaactcaaagagcaatacgctgcggaaag 1504
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
X62909:   1444 atgaagcaattggtgctgatcacagatgataaaactcaaagagcaatacgctgcggaaag 1503
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Fig. 1. Resequencing of the plasmid pPyrD ARA containing the cDNA of *A. thaliana* DHODH (acc. no. X62909). Partial alignment of the sequence established in this study (acc. no. AF454729) and the previously published sequence from X62909 [23]. The premature and the correct stop-codon as well as the additional thymidine, 't', are marked in gray.

3.3. Kinetic characterization and inhibition of *A. thaliana* DHODH

Since activity measurements of the recombinant full-length enzyme across a wide range of pH (5.5–9.0) revealed a maximum of activity at pH 7.5 (data not shown), all assays were performed at this value. From the characteristic bell shaped activity profile, two pK_a values (pK_{a1} = 6.5; pK_{a2} = 8.8) could be calculated. For comparison, the optimum of the vertebrates and insect species was at pH 8.0 [19,24,28].

When tested with Q_D as electron acceptor, the k_{cat} value for the truncated DHODH was found to be considerably higher than that for the full-length enzyme (Table 1), whereas the flavin content of the two proteins was similar (AT-DHODH: 0.8–1.1 mol flavin/mol protein; Δ75AT-DHODH: 0.6–0.9 mol flavin/mol protein). From these data it can be concluded that the difference in k_{cat} was not due to a different content of the cofactor FMN in the purified proteins. Rather, it is not unreasonable to assume that the long N-terminal mitochondrial targeting sequence of 57 amino acids – in comparison to 10 amino acids of the mammalian species – could be of unfavorable influence on the correct folding and stability of the protein over-expressed in *E. coli*. This assumption can be concluded from the lower expression rate obtained for the full-length plant enzyme in our study, as well as for the full-length *P. falciparum* DHODH in contrast to the appropriate N-terminal truncated mutant (G. McConkey, University of Leeds, personal communication on first experiments). For comparison, the expression rate and k_{cat} values of the purified mammalian DHODH did not give the same pattern when comparing the full-length and truncated versions (e.g. human: 107 s^{-1} and 75 s^{-1}, respectively [19]).

A non-classical two-site ping-pong mechanism, typical of an enzyme that contains two non-overlapping and kinetically isolated substrate binding sites, has been described with DHODH isolated from bovine liver [25]. When our data for AT-DHODH were plotted in double-reciprocal form, the typical pattern of parallel straight lines for this kind of mechanism was obtained for the co-substrate Q_D (not shown). As can be seen from Table 1, the K_m values (40 μM DHO for AT-DHODH; 121 μM DHO for Δ75AT-DHODH) were noticeably higher than those of the full-length and truncated mammalian enzymes (e.g. human: 6 μM and 9 μM [19,24]). An even more pronounced difference was obtained for Q_D. Whereas the K_m value of both versions of the human enzyme was 14 μM, that of the the full-length plant enzyme was 112 μM, and of the truncated plant DHODH it was 341 μM with this quinone.

Here, we compared the activity of AT-DHODH and Δ75AT-DHODH with a variety of quinone acceptors (Table 2). Q_{10} is present in all parts of the plant whereas plastoquinone and menaquinones (vitamin K1 and K2) seem to occur only in green tissues [29]. The endogenous electron acceptor of AT-DHODH is not known. Because the long isoprene side-chain of natural quinones caused solubility problems in enzyme assays, they often were replaced by the appropriate quinones without side chain or by Q_D since this

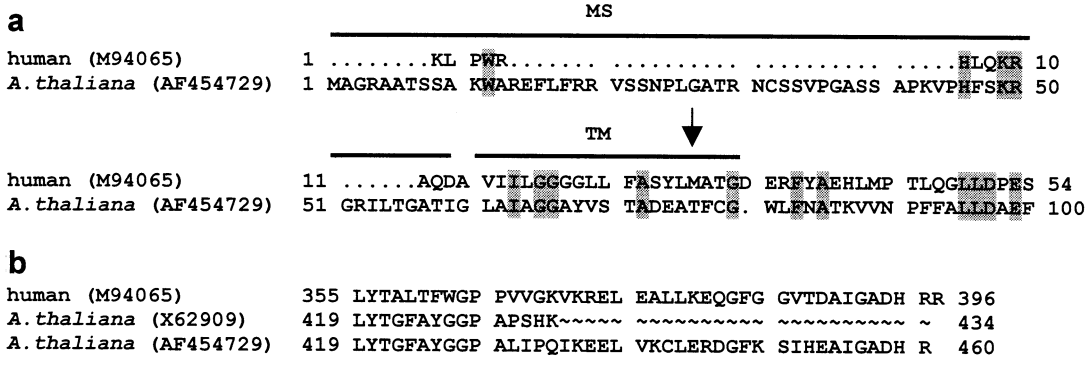

Fig. 2. a: Partial alignment of the N-termini of human and *A. thaliana* DHODH. Conserved amino acids are marked in gray. MS: Mitochondrial targeting sequence, TM: hydrophobic transmembrane sequence. b: Partial alignment of the predicted C-terminal amino acid sequence of human and *A. thaliana* DHODH. AF454729: Established in this study. X62909: Sequence communicated by Minét et al. [23]. M94065: Human DHODH.

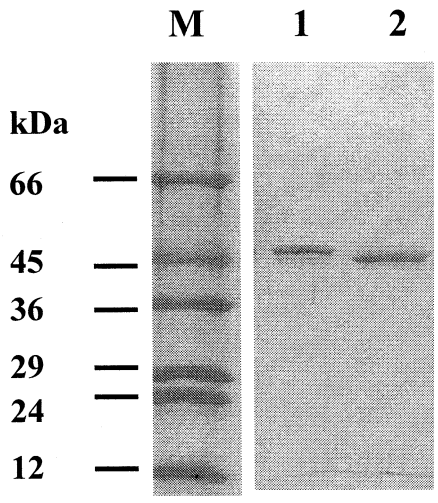

Fig. 3. SDS–PAGE of AT-DHODH-8His and Δ75AT-DHODH-8His purified on Ni^{2+}-NTA chromatography. M: Molecular mass marker. Lane 1: Δ75AT-DHODH-8His. Lane 2: AT-DHODH-8His (2 μg protein per lane).

has been commercially available. As previously reported for the animal DHODH [24,28], under the chosen test conditions Q_D was found to give an elevation of the plant DHODH activity in comparison to the natural Q_{10}; PQ_D was similar to Q_D. Interestingly, the replacement of Q_D by Q_0 or PQ_0 (Table 2) revealed a comparable or even enhanced activity of the full-length plant enzyme, but not of the truncated one. The same tendency was seen with menadione (vitamin K3) at higher concentration. For comparison, the activity of the full-length insect DHODH activity was found to be reduced by 20–60% on addition of these quinones as alternative electron acceptors [29]. Similar observations were described when Q_0, vitamin K1 or menadione were tested instead of Q_{10} or Q_6 with the isolated human or bovine enzyme [14,25]. These findings point to a considerable interference of the long N-terminal mitochondrial targeting sequence of the AT-DHODH (57 amino acids versus 19 of the insect and 10 of the human enzyme) with the side chain of the quinone. Whereas human, rat and insect DHODH were shown to be not proteolytically processed during import in mitochondria in vivo and in vitro [9], it is not known for the plant DHODH whether the N-terminal segment with the mitochondrial-targeting presequence is cleaved off or not.

In plants, strategies of limiting the pyrimidine nucleotide pools with the rationale of growth manipulation have not received much attention to date. Rather, the metabolic inhibitors 5-fluorouracil, 5-fluoroorotic acid and *N*-phosphonoacetyl-L-asparate, which are toxic to plant cells just as to animal cells [5,6], have been used as tools to understand the regulation and expression of pyrimidine biosynthesis in plants. Because specific inhibitors for DHODH of these organisms have not been described, we investigated the recombinant plant enzyme for its susceptibility to two compounds already proven as potent inhibitors of the mammalian DHODH in the nanomolar range: brequinar, originally developed as cytostatic agent [14] and A77-1726 with anti-inflammatory efficacy profile [12]. The present study revealed only a marginal inhibitory effect on *A. thaliana* DHODH: in the presence of 100 μM A77-1726, the activity of the full-length DHODH was reduced only by 10%, that of the truncated one was reduced by 25%. The residual activity of both enzymes was 90–95% in the presence of 100 μM brequinar. This negligible effect of brequinar may be explained by sequence and structure analysis of DHODH. The high resolution crystal structure that has been provided for an N-terminal truncated human DHODH in complex with brequinar [18] reveals key interactions that could govern effective binding of this compound. First, the carboxylate hydrogen of the brequinar molecule bonds to the side chain of Gln47 and a water molecule forms hydrogen bonds with the carboxylate and the γ-oxygen of Thr360. Gln47 of the human enzyme is replaced by Phe92 in AT-DHODH and Thr360 is replaced by Ala425. Second, the biphenyl group of the drug was shown to have four hydrophobic contacts with side chains of amino acids, one of which, Leu68, is replaced by Val113 in the plant enzyme. These differences in the human and plant protein could be responsible for the marginal effect observed here with this cinchoninic acid derivative. In contrast, Arg136, which gives a salt bridge

Table 1
Kinetic constants of the purified truncated and full-length *A. thaliana* DHODH

	AT-DHODH	Δ75AT-DHODH
k_{cat} (s^{-1})	8.2	72.9
Q_D		
K_m (μM)	112 ± 26	341 ± 99
k_{cat}/K_m (M^{-1} s^{-1})	73 214	213 783
DHO		
K_m (μM)	40 ± 10	121 ± 37
k_{cat}/K_m (M^{-1} s^{-1})	205 000	602 479

The concentration of DHO was varied from 5 to 500 μM at five fixed concentrations of Q_D (30, 50, 100, 200, 500 μM). The concentration of Q_D was varied from 30–500 μM at five fixed concentrations of DHO (5, 10, 50, 200, 500 μM). Results are means ± S.D. from three measurements. The k_{cat} values were calculated using the equation $V_{max} = k_{cat} \times [E]$, where $[E]$ = total enzyme concentration and is based on one binding site/monomer for each of the both substrates.

Table 2
Use of different quinone electron acceptors by recombinant *A. thaliana* DHODH

Electron acceptor	100 μM		500 μM	
	AT-DHODH	Δ75AT-DHODH	AT-DHODH	Δ75AT-DHODH
Q_D	100	100	100	100
Q_0	102 ± 10	67 ± 8	129 ± 17	67 ± 9
PQ_0	95 ± 23	75 ± 10	118 ± 24	66 ± 17
Q_{10}	83 ± 12	76 ± 17	n.d.	n.d.*
PQ_D	95 ± 7	104 ± 5	68 ± 2	86 ± 6
menadione**	82 ± 1	78 ± 11	103 ± 9	74 ± 18

Specific activities observed in the presence of different quinone derivatives are expressed relative to activity in the presence of Q_D (set as 100%). Quinone derivates were tested at 100 μM and 500 μM with the standard chromogen reduction assay. Results are expressed as mean percentage activity ± S.D. ($n = 8$, 100 μM; $n = 5$, 500 μM). (*) Not determined, solubility problems; (**) $n = 5$.

to brequinar, and Tyr356 form hydrogen bonds with A77-1726 in the human enzyme. Both amino acids are conserved at the respective position in the AT-DHODH sequence, Arg181 and Tyr421. Since a faint susceptibility of the plant enzyme to A77-1726 was observed, studies with other representatives of this class of compounds would be necessary to state more precisely the isoxazole suitability as a lead for development of specific inhibitors.

3.3.1. Concluding remarks. This is the first study and characterization of a purified recombinant plant DHODH, an enzyme that occupies a central position in de novo pyrimidine biosynthesis and that has become a focal point for clinical and therapeutical manipulation of the pathway in humans, animals and pathogens [30]. In addition, DHODH from agronomically important fungi has been identified as a new antifungal target [21]. Only recently, the differences in responses of insect DHODH and the enzyme from other species have been recognized as promising for the design of new agents that could selectively control insect growth due to pyrimidine nucleotide limitation thus contributing to animal and plant health [28]. With respect to the symbiosis of plants, insects and fungi and also in view of the immense importance of intact pyrimidine metabolism for the production of polysaccharides and secondary metabolites in plants, we now bring in the plant DHODH; this to be exploited as putative target enzyme in the development of novel growth control strategies, but also to be considered as vulnerable host when compounds are administered to combat parasites, pathogenes and insects.

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