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Palle Gravgaard Sørensen · Gert Dandanell

A new type of dihydroorotate dehydrogenase, type 1S, from the thermoacidophilic archaeon *Sulfolobus solfataricus*

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Abstract Dihydroorotate dehydrogenase (DHOD) (EC 1.3.3.1) from the thermoacidophilic archaeon *Sulfolobus solfataricus* P2 (DSM 1617) was partially purified 3,158-fold, characterized, and the encoding genes identified. Based on enzymological as well as phylogenetic methods, dihydroorotate dehydrogenase from *S. solfataricus* (DHODS) represents a new type of DHOD, type 1S. Furthermore, it is unable to use any of the (type-specific) natural electron acceptors employed by all other presently known DHODs. DHODS shows optimal activity at 70°C in the pH range 7–8.5. It is capable of using ferricyanide, 2,6-dichlorophenolindophenol (DCIP), Q_0, and molecular oxygen as electron acceptor. Kinetic studies employing ferricyanide indicate a two-site ping-pong mechanism with K_M values of 44.2 ± 1.9 μM for the substrate dihydroorotate and 344 ± 21 μM for the electron acceptor ferricyanide, as well as competitive product inhibition with a K_i of 23.7 ± 3.4 μM for the product orotate (OA). The specific activity, as determined from a partially purified sample, is approximately 20 μmol mg^{-1} min^{-1}. DHODS is a heteromeric enzyme comprising a catalytic subunit encoded by *pyrD* (291 aa; MW = 31.1 kDa) and an electron acceptor subunit (208 aa; MW = 23.6 kDa), encoded by *orf1*. DHODS employs a serine as catalytic base, which is unique for a cytosolic DHOD. To our knowledge, this work represents not only the first study on an archaeal DHOD but the first on a nonmesophilic DHOD as well.

Key words Dihydroorotate dehydrogenase · *Sulfolobus solfataricus* · Purification · Characterization · Sequence analysis

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P.G. Sørensen · G. Dandanell (✉)
Department of Biological Chemistry, Institute of Molecular Biology, University of Copenhagen, Sølvgade 83 H, 1307 Copenhagen K, Denmark
Tel. +45-3532-2025; Fax +45-3532-2040
e-mail: dandanell@mermaid.molbio.ku.dk

Introduction

The flavoenzyme dihydroorotate dehydrogenase (DHOD) catalyzes the conversion of 5,6-dihydroorotate to orotate in a redox reaction, which is the fourth of six universally conserved enzymatic reactions in the pyrimidine (UMP) de novo synthetic pathway. Phylogenetic studies of DHOD sequences show that they are divided into two major classes (Jensen and Björnberg 1998). This partition is reflected in the properties of the enzymes. The class 1 enzymes are cytosolic and found in gram-positive bacteria and Archaea, whereas class 2 enzymes are membrane attached and are found in gram-negative bacteria and eukaryotes. Traditionally, class 1 is divided into two subtypes. The type 1A enzyme, which uses fumarate as electron acceptor, is a dimer encoded by the *pyrD* gene whereas the type 1B enzyme, employing NAD^+ as electron acceptor, is a heterotetramer encoded by the *pyrD* and the *pyrK* (*pyrDII*) genes. The class 2 enzyme is a monomer that, in gram-negative bacteria, is attached to the membrane by an N-terminal three-helix structure acting as a suction disc. In addition, eukaryotic DHODs seem to contain a signal anchor in the extreme N-terminal, guiding it to the mitochondrial intermembrane space and embedding it in the inner membrane with an additional transmembrane helix. The suction-disc structure found in all class 2 DHODs is currently believed to be the binding site for respiratory quinones, connecting the enzyme to the respiratory chain and facilitating the use of molecular oxygen as terminal electron acceptor (Liu et al. 2000).

Sulfolobus solfataricus, which is a thermoacidophilic archaeon of the archaeal kingdom Crenarchaeota, has been chosen as the model organism for studies on thermophilic Archaea. *S. solfataricus* is able to grow in the temperature range 60°–90°C in the pH range 3–5 with optimal growth at 83°C, pH 3.5 in sulfuric acid. The size of the circular genome is 3 Mbp and the G+C content is 36%.

Materials and methods

Microorganism

The thermoacidophilic archaeon *Sulfolobus solfataricus* P2 (DSM 1617), obtained from the German collection of microorganisms (DSM), was a generous gift from Prof. N. Glansdorff, Brussels, Belgium.

Enzyme assay and protein determination

A 640-µl assay mix (86 mM Tris-HCl, pH 8.5, 0.8 mM $K_3[Fe(CN)_6]$ (III), with and without 1 mM dihydroorotate, DHO) and a 10-µl sample were mixed in Eppendorf tubes, preincubated for 5 min at 70°C, and incubated at 70°C for 1 h. After incubation, one volume of 1 M perchloric acid (PCA) was added to stop the reaction and to precipitate protein. After centrifugation at 4°C for 5 min in a microcentrifuge, the absorbance of the supernatant was monitored at 290 nm (direct product formation) and 420 nm (depletion of electron acceptor) in a Beckman spectrophotometer (Fullerton, CA, USA). Protein concentration was determined by the bicinchonic acid (BCA) method using the standard assay kit from Pierce (Rockford, IL, USA) with bovine serum albumin as standard; 1 unit is defined as 1 nmol orotate produced per minute. Activity was calculated from 420-nm measurements exclusively with 290-nm measurements as the internal control. The extinction coefficient at 420 nm for the reduction of $K_3[Fe(CN)_6]$ (III) is 1,020 M^{-1} cm^{-1} (Jordan et al. 2000). Consequently, the $\varepsilon 420_{OA}$ for orotate formation is 2,040 M^{-1} cm^{-1}. The extinction coefficient of $K_3[Fe(CN)_6]$ at pH 7.0 and in PCA differs less than 5%.

Partial purification of dihydroorotate dehydrogenase from *Sulfolobus solfataricus*

Precipitations were made on ice, and all centrifugations (Sorvall SS-34; DuPont, Wilmington, DE, USA), dialyses, and columns, except the MonoP column, were run at 4°C. The cell pellet from 39 g *S. solfataricus* was resuspended on ice in 240 ml 50 mM imidazole citrate buffer (pH 6.5) (IC buffer), and cells were disrupted by sonication [60 cycles of 30 s sonication at 44 µm amplitude followed by a 30-s break with a Sanyo MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK) using a 9.5-mm probe]. Cell debris was removed by centrifugation (5,000 *g*, 10 min), and the supernatant was used as the crude extract for the purification.

As an initial centrifugation at 14,500 *g* caused up to 30% loss of activity, a stepwise centrifugation (10,500 *g*, 30 min, and 14,500 *g*, 30 min) was performed before addition of streptomycin sulfate. Streptomycin sulfate was added to a final concentration of 0.8%, incubated on ice for 1 h, and centrifuged (14,500 *g*, 30 min). The supernatant was dialyzed overnight against IC buffer (pH 6.5) and centrifuged (14,500 *g*, 30 min). Next, dihydroorotate dehydrogenase from *S. solfataricus* (DHODS) was fractionated with polyethylene glycol 4000 (PEG4000); 45% PEG4000 (w/v) in 100 mM Tris-HCl (pH 7.5) was added to the supernatant to a final concentration of 5%, incubated on ice for 1 h, and centrifuged (8,000 *g*, 10 min, and 10,000 *g*, 10 min). Then, PEG4000 was added to the supernatant to a final concentration of 15%, incubated on ice for 1 h, and centrifuged (6,000 *g*, 10 min, and 10,000 *g*, 10 min). The pellet was resuspended in a total 120 ml 10 mM Tris-HCl (pH 7.5) and applied to a 6 ml Resource Q column (Pharmacia; Amersham Pharmacia Biotech, Buckinghamshire, UK) in aliquots corresponding to approximately 1 g protein in crude extract. After washing with the same buffer, the proteins were eluted by a 0–1 M NaCl gradient. Samples containing activity were pooled, after which Na_2SO_4 was added to a final concentration of 0.5 M and applied on a 25 ml phenyl sepharose column (Pharmacia Phenyl Sepharose 6 Fast Flow, high substitution), equilibrated in 0.25 M $(NH_4)_2SO_4$, 10 mM Tris-HCl (pH 7.5). A gradient from 0.25 M $(NH_4)_2SO_4$, 10 mM Tris-HCl (pH 7.5) to 100 mM Tris-HCl (pH 7.0) was run, and the column was washed with 5 vol buffer before elution of sample in a step gradient to glass distilled water (GDW). Before elution, 1/10 vol 100 mM Tris-HCl (pH 8.5) was added to the collection tubes.

Fractions containing activity were pooled and dialyzed overnight against 10 mM Tris-HCl (pH 8.5). The dialyzed sample was applied on a 6-ml MonoQ column (Bio-Rad Uno-Q; Bio-Rad, Hercules California, USA), washed with 5 vol 10 mM Tris-HCl (pH 7.5), and eluted in a gradient of 10 mM Tris-HCl (pH 7.5) to 1 M NaCl in 25 mM Tris-HCl (pH 8.5). Fractions containing activity were pooled and dialyzed 3 × 1 h against IC buffer (pH 6.5), applied on a 10-ml Red Sepharose column (Amicon Red; Amicon, Beverly, MA, USA) and eluted in a gradient of 10 mM IC buffer (pH 6.5) to 1 M NaCl in 25 mM Tris-HCl (pH 8.5). Pooled fractions (60 ml) were concentrated to approximately 6 ml in centrifugation spin columns (Amicon; Centriprep), dialyzed overnight in IC buffer (pH 6.5), and applied on a 6-ml Blue Sepharose column (Pharmacia; Blue CL-6B), and eluted as on the red column. Pooled fractions were concentrated to approximately 1 ml in centrifugation spin columns (Amicon; Centriprep), followed by buffer change to 100 mM bis-Tris buffer (pH 7.6) using Microcon centrifugation spin columns (Amicon; 10 kDa). Finally, the sample was applied on a 1-ml MonoP HR 5/5 (Pharmacia) column for chromatofocusing in the pH interval 5.5–6.5 using polybuffer 94/96. Chromatofocusing was performed at room temperature according to the manufacturer's instructions. Fractions containing highest activity were pooled, and the buffer was changed to 10 mM Tris-HCl (pH 8.5) and concentrated to approximately 0.5 ml using Microcon centrifugation spin columns. This fraction was used for characterization.

Protein electrophoresis

SDS-PAGE (12.5% slab gel; Mini Protean II system, Bio-Rad) was performed by the procedure of Laemmli (1970). Protein bands were stained with Coomassie brilliant blue G 250. The molecular weight marker was Benchmark Protein Ladder (Gibco, Rockville, IN, USA).

pH optimum experiment

DHODS activity was assayed at different pHs in two different polybuffers: a "universal buffer" consisting of 5,5-diethylbarbituric acid, citric acid, and KH_2PO_4 (15 mM, adjusted to the desired pH with NaOH), and a Tris-based polybuffer consisting of 2-[*N*-morpholineo]ethanesulfonic acid (MES)-Tris-glycine (0.1 M). All pH values were measured at 70°C because both buffer systems are temperature dependent.

Electron acceptor experiment

Assays employing various electron acceptors (final concentration, 0.8 mM) were essentially performed as already described but at different wavelengths. Extinction coefficients employed in electron acceptor screening corresponding to orotate (OA) formation were O_2 (280 nm, ε = 7,500 M^{-1} cm^{-1}), $K_3[Fe(CN)_6]$ (III) (420 nm, ε = 2,040 M^{-1} cm^{-1}), Q_0 (287 nm, ε = 6,000 M^{-1} cm^{-1}), fumarate (280 nm, ε = 7,240 M^{-1} cm^{-1}), NAD^+ (340 nm, ε = 6,200 M^{-1} cm^{-1}), and DCIP (297 nm, ε = 3,400 M^{-1} cm^{-1}) (Björnberg et al. 1999; Karibian 1978).

Results

Purification and identification of DHODS subunits

The final partial purification of DHOD from *S. solfataricus* is summarized in Table 1. The enzyme was purified 3,158-fold with a recovery of 3.3% in nine steps. As seen in lane 10 of Fig. 1, the highly enriched sample is not pure. To identify DHODS among the approximately ten bands seen on SDS-PAGE, samples were applied on gel filtration and chromatofocusing columns (data not shown). By comparing the intensity of the bands on SDS gels with the DHODS activity of the fractions eluted from both columns, two bands were found to correlate. These two bands were submitted to N-terminal sequencing. The larger band around 31 kDa in the gel showed an N-terminal sequence of MITIKN, corresponding to the putative *pyrD* gene (31.1 kDa, 291 aa) found by homology by the *S. solfataricus* genome project (accession number, CAB57690). The N-terminal sequence of the smaller band around 26 kDa, MIYYEVIEKRKNVDN, was also found in the database, but as an unassigned *orf1* (23.6 kDa, 208 aa) of the *pyr* operon (accession number CAB57688). This result strongly indicates that DHODS consists of two different subunits. It should be noted that the lower band around 26 kDa in lane 10 is a double band of approximately equal intensities that is poorly resolved in this gel. The band that overlaps ORF1, which is very intense in lane 9, is presumably a degradation product of the intense band around 40 kDa seen in lane 8.

Specific activity, T_{opt}, pH_{opt}, and pI

By visual inspection and scanning (Kodak 1D analysis software) of SDS-PAGE gels (Fig. 1, lane 10), the two bands (encoded by *pyrD* and *orf1*) correspond to approximately 20% of the remaining protein; this means that the specific activity of DHODS is estimated to be 20 μmol mg^{-1} min^{-1}. We also tested the temperature and pH dependency and found the maximal enzymatic activity at 70°C (Fig. 2A) in the pH range 7.0–8.5 (Fig. 2B). The native pI, as determined by isoelectric focusing (IEF) gels and chromatofocusing, is 6.0 ± 0.2 (data not shown).

Analysis of the kinetic mechanism

Kinetic constants were determined for both the substrate dihydroorotate (DHO) and the electron acceptor ferricyanide as well as for the reaction product orotate. When

Table 1. Purification of dihydroorotate dehydrogenase (DHOD) from *Sulfolobus solfataricus*

Step	Total protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)	Yield (%)	Purification (fold)
1. Crude extract[c]	6,000	7,501	1.3	100[d]	1
2. Streptomycin precipitation	4,572	9,234	2.0	123	1.5
3. PEG precipitation	2,202	8,237	3.7	110	3.0
4. Resource Q	281	5,612	20	75	16
5. Phenyl sepharose	55.2	5,367	97	72	78
6. MonoQ	21.7	4,732	218	63	174
7. Red sepharose	2.33	1,500	645	20	516
8. Blue sepharose	0.52	722	1,392	9.6	1,113
9. MonoP chromatofocusing	0.06	249	3,948	3.3	3,158

PEG, polyethylene glycol

[a] Protein concentrations were determined in triplets by the Pierce BCA protein Assay Kit with BSA as standard

[b] 1 unit=1 nmol orotate produced/min

[c] Cells used were about 39 g (wet weight) corresponding to a 25-l culture

[d] The activity in crude extract is very uncertain because of high background activity depleting the electron acceptor; this depletion will lower the measured activity considerably. The apparent increase in total activity in the first purification steps has been observed on various occasions, and is consistent with the loss of background activity

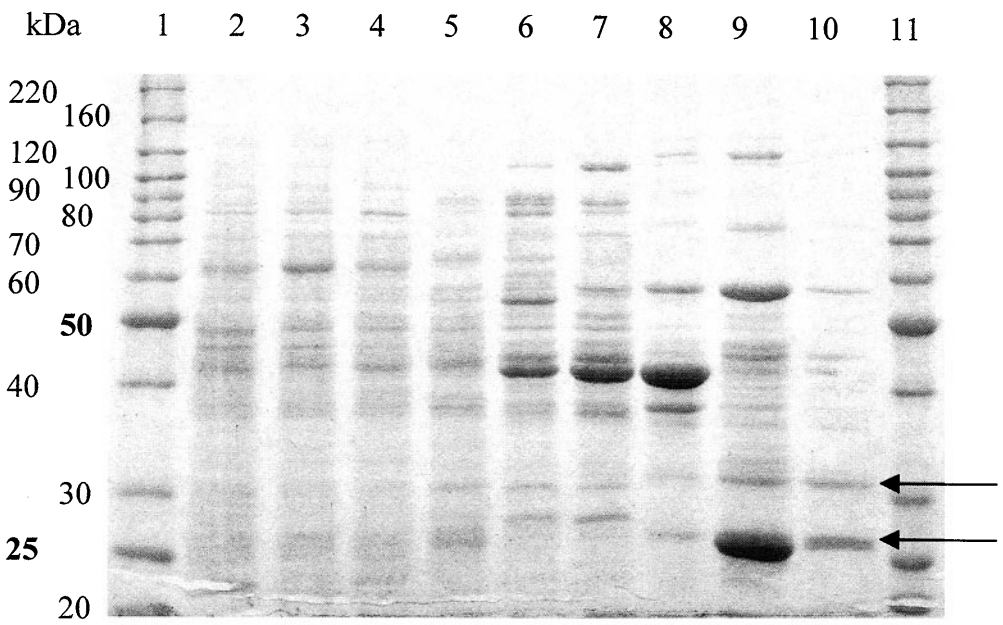

Fig. 1. The 12.5% SDS-PAGE of the partial purification. *Lanes 1, 11*, benchmark protein ladder marker (5 µl); *lanes 2–10*, purification steps as described in Table 1 ; *lanes 2–4*, 50× diluted sample; *lanes 5, 6*, 10× diluted sample; *lane 7*, 5× diluted sample; *lanes 8–10*, undiluted sample. Volume of all samples, 10 µl. *Arrows* indicate the two bands subjected to N-terminal sequencing

the concentration of DHO was varied in the presence of different, fixed concentrations of the electron acceptor ferricyanide, a series of parallel lines were obtained in a double-reciprocal plot of reaction velocity versus substrate concentration (Fig. 3). This velocity pattern indicates that DHODS, similar to many other flavoenzymes, follows a ping-pong type of mechanism. K_M values for DHO and for ferricyanide were 44.2 ± 1.9 µM and 344 ± 21 µM, respectively. To further elucidate the mechanism, inhibition by the reaction product orotate was analyzed. When the electron acceptor concentration was kept constant, the addition of orotate increased the apparent K_M values for DHO but left the apparent V_{max} values unaffected (Fig. 4). This result shows that orotate is a competitive inhibitor (K_{is} = 23.7 ± 3.4 µM) with respect to DHO, indicating that there is no competition between orotate and the electron acceptor, because such a competition would have caused a reduction of the apparent V_{max} values in the presence of orotate (a K_{ii} value of 2.7×10^{27} µM was obtained when data were fitted to noncompetitive inhibition). These results strongly indicate that, when using ferricyanide as electron acceptor, DHODS works by a two-site ping-pong mechanism with one binding site for DHO and orotate and a different binding site for oxidized and reduced electron acceptor.

Electron acceptor specificity

To identify which electron acceptors DHODS can use, we tested a series of natural and synthetic electron acceptors. DHODS is able to use ferricyanide, Q_0, DCIP, and molecular oxygen as electron acceptor (Table 2). It is not, however, able to employ any of the natural electron acceptors used as one of the hallmarks for the division of DHOD into classes and types. Consequently, the natural electron acceptor for DHODS is still unknown.

Table 2. Screening for electron acceptors

Compound added to assay	Activity (relative to oxygen)
Protein sample (oxygen as acceptor)	1[c]
$K_3[Fe(CN)_6]$ (III)	14.5
Q_0	8.7
Fumarate	<0.1
NAD^+	0.1
Coenzymes Q_6 and Q_{10}[a]	<0.1
DCIP[b]	16.7

[a]These results were obtained using crude extract (Sørensen and Dandanell 1999)
[b]DCIP is seemingly the best electron acceptor; at high temperatures, however, it precipitates reversibly, giving irreproducible results
[c]The specific activity with oxygen as acceptor is 272 U/mg

Active site base

Until the discovery of two *pyrD* sequences from two *Sulfolobus* species, *S. solfataricus* (accession number CAB57690) and *S. acidocaldarius* (accession number O08358), respectively, all class 1 DHODs were found to employ a cysteine as catalytic base whereas class 2 enzymes employed a catalytic serine. To substantiate the finding of a catalytic serine in a class 1 DHOD, an iodoacetamide (IAAM) inactivation experiment was conducted on DHODS. IAAM has been used to irreversibly inactivate the catalytic cysteine in class 1 DHODs by oxidation. A prolonged incubation with IAAM did not inactivate DHODS although total inactivation of the control enzyme (class 1, DHODB from *Lactococcus lactis*) was detected (data not shown). The lack of inactivation of DHODS indicates that the active site base is not a normal class 1 cysteine, thereby corroborating the serine found in the sequences.

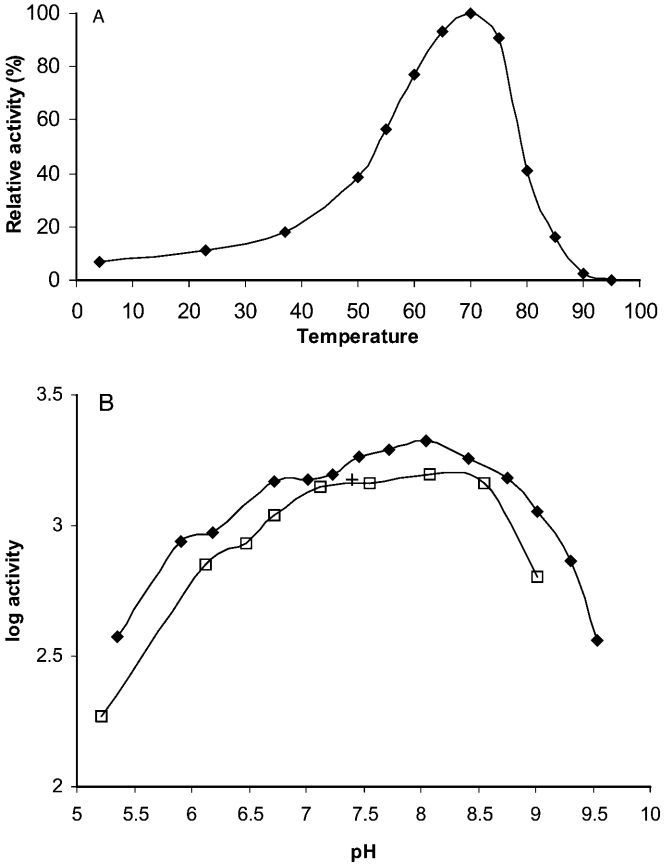

Fig. 2. Effect of temperature (**A**) and pH (**B**) on the activity of dihydroorotate dehydrogenase from *S. solfataricus* (DHODS). **A** The optimal assay temperature employing an incubation period of 1 h is 70°C. Because of instability of the enzyme, this optimal temperature is highly dependent on the incubation period: the shorter the period, the higher the optimal temperature. **B** Two different polybuffers were employed in this analysis: a "universal buffer" (*diamonds*) and a Tris-based polybuffer (*squares*). For comparison, the activity measured in standard assay buffer is marked with a *cross*. DHODS has detectable activity in the pH interval 5–9.5 with a plateau at pH 7–8.5

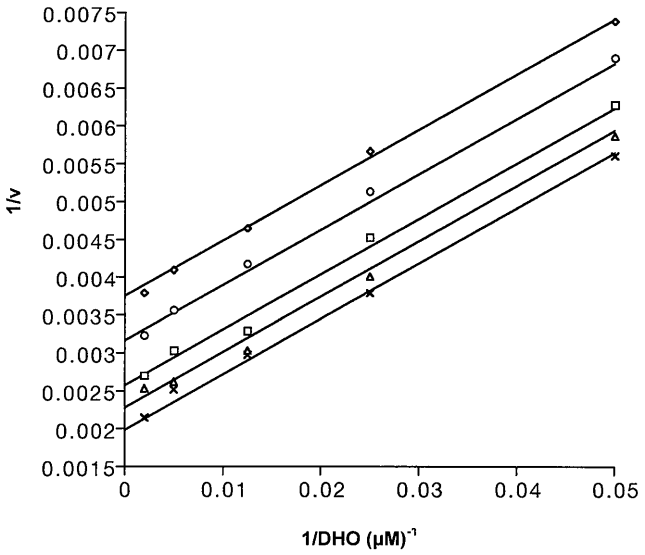

Fig. 3. Velocity pattern varying dihydroorotate (DHO) and $K_3[Fe(CN_6)]$ (III). The experiment was conducted in a 5 × 5 matrix using 20, 40, 80, 200, and 500 μM DHO at five concentrations of $K_3[Fe(CN)_6]$ (III): 250 μM (*diamonds*), 333 μM (*circles*), 500 μM (*squares*), 667 μM (*triangles*), and 1 mM (*crosses*). Application of the program of Cleland (1971) gave K_M values for DHO and ferricyanide of 44.2 ± 1.9 μM and 344 ± 21 μM, respectively

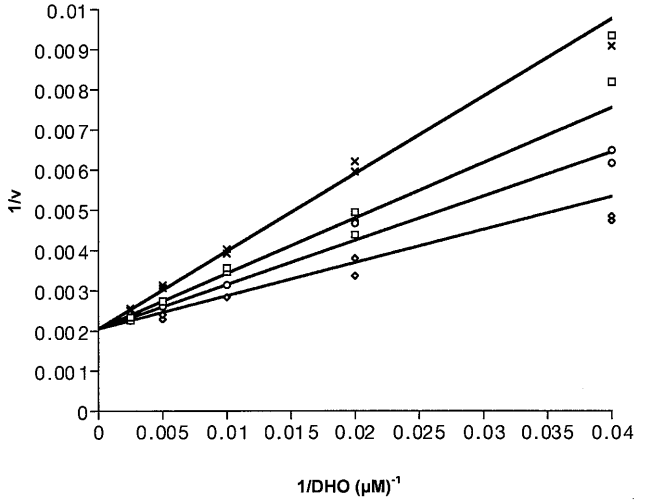

Fig. 4. Inhibition of DHODS by orotate. The effect of orotate, 0 μM (*diamonds*), 10 μM (*circles*), 20 μM (*squares*), and 40 μM (*crosses*) while varying the concentration of DHO (25, 50, 100, 200, and 400 μM). As seen by the common intersection of the *y*-axis (unaltered V_{max}), orotate exhibits competitive inhibition with respect to DHO. Application of the program of Cleland (1971), assuming noncompetitive inhibition, gave a K_{is} value of 23.7 ± 3.4 μM (and a K_{ii} of 2.7×10^{27}) and a K_M value of 37.5 ± 4.8 μM for DHO

Discussion

The distinctive traits of the DHOD classes and types currently known are shown in Table 3. It must be emphasized that this division is strictly biochemical and, although the division is reflected in phylogenetic studies of the catalytic subunit (Jensen and Björnberg 1998), none of the divisions are believed to correspond to different ancestry. Consequently, we have chosen to use the term "class" instead of the term "family" currently used in the literature, because in bioinformation terms families are defined by common ancestry and consequently two DHOD families could only arise through convergent evolution.

Based on the distinctive traits described in this article, we propose that dihydroorotate dehydrogenase from *Sulfolobus solfataricus* (DHODS) is a new type, type 1S. As the original division into class 1 and 2 corresponded to the cellular localization and DHODS is found in the cytosol, it is defined as a class 1 DHOD. Furthermore, unrooted phylogenetic trees show that class 1 and 2 sequences are readily separated on the basis of the class division (Jensen and Björnberg 1998). The two *Sulfolobus* sequences align, although not deeply, in class 1 as a separate branch in the proximity of the type 1B branch (P.G. Sørensen, in preparation).

Table 3. Properties of the different DHOD classes and types

	Cellular localization	Subunit composition[a]	Active site base	Prosthetic groups	Natural electron acceptor
Type 1A	Cytosol	D_2	Cys	FMN	Fumarate
Type 1B	Cytosol	D_2K_2	Cys	FMN, FAD, [FeS]	NAD^+
Class 2	Membrane	D	Ser	FMN	Resp. quinones
Type 1S	Cytosol	(D_2X_2)	Ser	FMN, [FeS], (FAD ?)	?

[a] K indicates a classical DHOD electron acceptor subunit encoded by the *pyrK* (*pyrDII*) gene; X indicates a new type of electron acceptor subunit encoded by *orf1* in *Sulfolobus*

That DHODS is a heteromeric enzyme is based on several indications. (a) The two proteins copurify with an activity that correlates with the intensity of the bands in SDS-PAGE from both gel filtration and chromatofocusing despite differences in size (31.1 and 23.6 kDa) and theoretical pI (6.4 and 8.2). (b) *orf1* is located in the *pyr* operon and contains a consensus sequence including four cysteines that in type 1B enzymes are used for fixation of a 2Fe–2S iron–sulfur cluster used in the transport of electrons from the active site to the electron acceptor site (Rowland et al. 2000). (c) Alignments show no sequence corresponding to a N-terminal suction-disc structure found in class 2 enzymes (Liu et al. 2000; Nørager et al., in preparation) and a structure prediction made on the catalytic subunit of DHODS corresponds to the triangular shape found in DHODs containing more than one subunit (data not shown). (d) Numerous attempts to express *pyrD* from *S. solfataricus* in *Escherichia coli* and yeast have been unsuccessful (data not shown); this result is in agreement with the finding that the catalytic subunit of DHODB from *Lactococcus lactis*, when expressed in *E. coli* without the electron acceptor subunit, is extremely unstable, with a half-life of 4 min at 25°C and 45 s at 37°C (Nielsen et al. 1996a).

We have not been able to determine the quaternary structure of DHODS in gel filtration experiments. DHODS elutes around the BSA marker (67 kDa), which is between a heterodimer and a heterotetramer (data not shown). Cross-linking experiments have been inconclusive because the preparation is not pure, although bands corresponding to homodimer, heterodimer, and heterotetramer structures were seen (data not shown). Cloning of the *pyrD* and *orf1* genes in *E. coli* is in progress. The finding of a catalytic subunit and an electron acceptor subunit places DHODS in the proximity of type 1B, but the inability to use NAD^+ as electron acceptor and a very low sequence similarity between *orf1* and *pyrK*, as well as the differences in catalytic base, necessitates a type distinction.

The approximate specific activity of 20 U/mg found for DHODS is well within the range of other specific activities found in the literature. The normal range between 15 and 120 U/mg is exemplified by 15 U/mg for the *Neurospora crassa* DHOD (Miller 1978), 17 U/mg for DHODA from *Lactococcus lactis* (Nielsen et al. 1996b), 48 U/mg for DHODB from *Lactococcus lactis* (Nielsen et al. 1996a), and 120 U/mg for DHODC from *E. coli* (Karibian 1978). The optimal pH range of 7–8.5 for DHODS is comparable to the optimal pH reported for other DHODs. An optimal pH range of 7.5–9 has been reported for DHODA (Nielsen et al. 1996b), pH 8 for DHODB (Nielsen et al. 1996a) and bovine liver DHOD (Hines et al. 1986), and a broad bell-shaped pH profile with a plateau in the pH range 7–8.5 for *Clostridium oroticum* DHOD (Argyrou et al. 2000). The pH optimum for DHODS is reasonable because, although the optimal environmental pH for *S. solfataricus* is 3.5, the internal pH is believed to be neutral (Dandanell, unpublished data).

From a thermophilic point of view, the optimal temperature of 70°C for DHODS is in the lower range of observed values, and the fact that 70°C is considerably higher than temperatures employed in DHOD studies (e.g., T_{opt} = 42°C for the *C. fasciculata* DHOD (Pascal and Walsh 1984) is not surprising because, to our knowledge, the results presented in this article represent the first work published on a DHOD from a thermophilic organism.

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