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Purification and characterization of dihydroorotate dehydrogenase A from *Lactococcus lactis*, crystallization and preliminary X-ray diffraction studies of the enzyme

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Abstract

Lactococcus lactis is the only organism known to contain two dihydroorotate dehydrogenases, i.e., the A- and B-forms. In this paper, we report the overproduction, purification, and crystallization of dihydroorotate dehydrogenase A. In solution, the enzyme is bright yellow. It is a dimer of subunits (34 kDa) that contain one molecule of flavin mononucleotide each. The enzyme shows optimal function in the pH range 7.5–9.0. It is specific for L-dihydroorotate as substrate and can use dichlorophenolindophenol, potassium hexacyanoferrate(III), and, to a lower extent, also molecular oxygen as acceptors of the reducing equivalents, whereas the pyridine nucleotide coenzymes (NAD^+, $NADP^+$) and the respiratory quinones (i.e., vitamins Q_6, Q_{10} and K_2) were inactive. The enzyme has been crystallized from solutions of 30% polyethylene glycol, 0.2 M sodium acetate, and 0.1 M Tris-HCl, pH 8.5. The resulting yellow crystals diffracted well and showed little sign of radiation damage during diffraction experiments. The crystals are monoclinic, space group $P2_1$ with unit cell dimensions $a = 54.19$ Å, $b = 109.23$ Å, $c = 67.17$ Å, and $\beta = 104.5°$. A native data set has been collected with a completeness of 99.3% to 2.0 Å and an R_{sym} value of 5.2%. Analysis of the solvent content and the self-rotation function indicates that the two subunits in the asymmetric unit are related by a noncrystallographic twofold axis perpendicular to the crystallographic *b* and *c* axes.

Keywords: evolution of dihydroorotate dehydrogenase; flavin; flavoprotein; FMN; pyrimidine nucleotide biosynthesis

Dihydroorotate dehydrogenase catalyzes the oxidation of dihydroorotate to orotate. The reaction constitutes the fourth step in the de novo biosynthesis of UMP (Neuhard, 1983). The enzyme was identified originally by Lieberman and Kornberg (1953) in extracts of the anaerobic bacterium *Zymobacterium oroticum* (now named *Clostridium oroticum*) in which it was present at high levels after growth with orotate as the sole source of carbon and energy. The dihydroorotate dehydrogenase of this organism is a soluble enzyme that couples the oxidation of dihydroorotate with the reduction of NAD^+ (Lieberman & Kornberg, 1953). However, subsequently discovered biosynthetic dihydroorotate dehydrogenases were all unable to utilize NAD^+ as a co-substrate (O'Donovan & Neuhard, 1970). The biosynthetic dihydroorotate dehydrogenase is attached to the cytoplasmatic membrane in the Gram-negative bacterium *Escherichia coli* (Karibian, 1978; Larsen & Jensen, 1985) and is located in the mitochondria in all eukaryotic organisms (see e.g., Pascal et al., 1983; Hines et al., 1986; Rawls et al., 1993; Angermüller & Löffler 1995), with the notable exception, however, of bakers yeast, in which the enzyme is cytosolic (Nagy et al., 1992; Roy, 1992).

Dihydroorotate dehydrogenase from *E. coli* is a dimeric enzyme consisting of identical subunits (338 amino acid residues), each containing one molecule of tightly bound flavin mononucleotide (Karibian, 1978; Larsen & Jensen, 1985). The protein shows very high sequence similarity (>40% identity) to all dihydroorotate dehydrogenases of mitochondrial origin, perhaps

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Abbreviations: FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; IPTG, isopropyl-β-D-thiogalactopyranoside; DCIP, dichlorophenolindophenol; UMP, uridine 5′-monophosphate.

because the mitochondria have evolved from a purple bacterium related to *E. coli* (Yang et al., 1985; Delihas & Fox, 1987). In contrast, the *E. coli* enzyme shows very little sequence similarity (<20% identity) with the corresponding enzymes from Gram-positive bacteria (*Bacillus subtilis* [Quinn et al., 1991], *B. caldolyticus* [Ghim et al., 1994] and *Lactococcus lactis* [Andersen et al., 1994]) whose potential membrane attachment is unknown, and with the cytosolic enzyme from bakers yeast (Roy, 1992).

Recently, it was found that the milk-fermenting bacterium *L. lactis* contains two genes (*pyrDa* and *pyrDb*) encoding functional dihydroorotate dehydrogenases (Andersen et al., 1994). Both of the enzymes seem to be of biosynthetic nature, because either of the corresponding genes, i.e., *pyrDa* and *pyrDb*, are able to complement the lack of dihydroorotate dehydrogenase in *E. coli* and because both of the genes must be inactivated by mutation in *L. lactis* in order to impose a pyrimidine requirement on the organism (Andersen et al., 1994). The two enzymes show little sequence similarity with each other (about 30% identity) even though both consist of polypeptides of 311 amino acid residues. Instead, one of the enzymes, dihydroorotate dehydrogenase A (encoded by *pyrDa*), is almost identical with the cytosolic dihydroorotate dehydrogenase from bakers yeast (i.e., the two proteins show approximately 71% sequence identity), whereas the other enzyme, dihydroorotate dehydrogenase B, resembles the dihydroorotate dehydrogenase from *B. subtilis*, the identity in an alignment of the two amino acid sequences being approximately 65% (Andersen et al., 1994).

We have undertaken a study of these two dehydroorotate dehydrogenases. In this paper, we describe the purification and initial characterization of dihydroorotate dehydrogenase A from *L. lactis* as well as crystallization conditions and preliminary X-ray diffraction data for this protein.

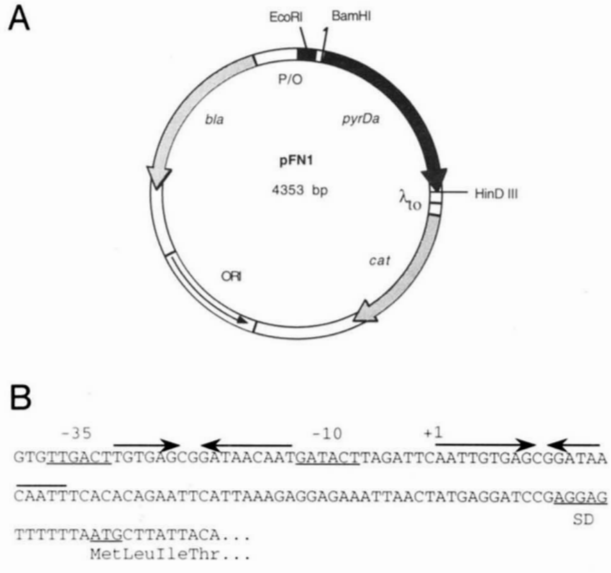

Fig. 1. Structure of (**A**) the expression plasmid pFN1 and (**B**) the nucleotide sequence of the promoter region. Transcription of the *pyrDa* gene is driven by the very strong $P_{A1/04/03}$ promoter, which is a synthetic derivative of the early A1 promoter of phage T7 containing two binding sites for the *lac*-repressor, as indicated by two pairs of opposite arrows in B (H. Bujard, pers. comm.). The promoter is indicated by P/O in A and by the −35 and −10 regions in B; bla, gene for β-lactamase; cat, gene for chloramphenicol acetyltransferase; ORI, origin of replication; +1, transcriptional start site; SD, ribosome binding site, and λ_{to}, transcription terminator of phage λ. Bottom line in B shows the N-terminal end of dihydroorotate dehydrogenase A.

Results and discussion

Preparation of dihydroorotate dehydrogenase A from L. lactis

In strain SØ6645, the very strong $P_{A1/04/03}$ promoter on the expression vector pFN1 (Fig. 1) is kept repressed by the *lacI* repressor, encoded by an F'*lacI*^{q} episome present in the cells. It is essential that the culture is grown to a reasonable density before induction with IPTG, because growth terminates after a few generations under inducing conditions due to a toxic effect of overexpressing dihydroorotate dehydrogenase. The purification procedure, described in the Materials and methods, gave an almost homogeneous enzyme preparation (not shown). The yield was 213 mg protein from 8 L of culture, corresponding to 43% of the initial content of dihydroorotate dehydrogenase in the crude extract (Table 1). The enzyme could be stored for several months without loss of activity at −20 °C in solutions containing 50% glycerol. However, when heated at 45 °C in the purification buffer, the enzyme lost half of the activity in 20 min.

SDS-gel electrophoresis revealed a subunit molecular mass of 34–36 kDa, in good agreement with the size of the protein chain as predicted from the sequence of the *pyrDa* gene, i.e., 34,188 Da (Andersen et al., 1994). During gel filtration chromatography on a Superose 6HR 10/30 column for the FPLC apparatus (Pharmacia) together with the MW-GF-200 marker protein kit from Sigma, the enzyme activity coeluted with bovine serum albumin (66 kDa). This indicates that dihydroorotate dehydrog-

Table 1. *Purification of dihydroorotate dehydrogenase A*

Purification step	Vol (mL)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (fold)
1) Crude extract	90	7,300	100	2	1
2) Streptomycin sup.	90	6,770	93	3	2
3) 1. DE52	200	4,607	63	8	5
4) 2. DE52	150	5,274	72	14	8
5) Hydroxylapatite and conc.	13	3,135	43	17	9

enase A from *L. lactis* is a dimeric enzyme as found for dihydroorotate dehydrogenase from *E. coli* (Larsen & Jensen, 1985).

The purified dihydroorotate dehydrogenase A has a bright yellow color and an absorption spectrum that is typical for an oxidized flavoprotein with absorption peaks at 372 nm and 457 nm. The flavin compound, released from the enzyme by treating with 0.25 M formic acid, co-migrated with FMN during chromatography on PEI-cellulose thin-layer plates and migrated twice as fast as FAD. Because a solution of 1 mg/mL enzyme shows an absorption $A_{457} = 0.29$, these results indicate that there is one molecule of FMN per subunit of dihydroorotate dehydrogenase A.

Optimal reaction conditions and substrate specificity

Dihydroorotate dehydrogenase A exhibited the highest reaction rates in the pH interval 7.5–9.0 (Fig. 2) using either DCIP (50 μM) or potassium hexacyanoferrate(III) (50 μM) as electron acceptors and L-dihydroorotate as substrate, while monitoring the production of orotate. Potassium hexacyanoferrate(III) gave rise to five times higher reaction rates than seen with DCIP, whereas molecular oxygen (about 0.2 mM) gave reaction rates that were four times lower than observed with DCIP. There was no detectable activity with fumarate, coenzymes Q6 or Q10, menaquinone, NAD^+, or $NADP^+$ as acceptors of the reducing equivalents. However, we found that the enzyme could catalyze an efficient interconversion of dihydroorotate and [^{14}C]-orotate, indicating that it works by a simple Ping-Pong reaction mechanism (Cleland, 1963). This is in contrast to the mammalian liver enzyme, which seems to act by a more complex two-site Ping-Pong mechanism (DeFrees et al., 1988; Hines & Johnson, 1989). The enzyme seemed to be specific for dihydroorotate as a substrate, because it was unable to convert dihydrouracil to uracil with DCIP as the electron acceptor. These characteristics indicate strongly that the enzyme is not a catabolic enzyme like the dihydropyrimidine dehydrogenases from bovine liver or *Pseudomonas* (Podschun et al., 1990; Lu et al., 1992; Yokata et al., 1994).

Crystallization and characterization by X-ray diffraction

An initial search for suitable crystallization conditions was carried out using the hanging drop vapor diffusion technique with the standard sparse matrix crystal screening solutions (Jancarik & Kim, 1991). Equal volumes of the crystallization buffer and a protein solution of 18 mg/mL (in 25 mM sodium phosphate, pH 6.0, with 10% glycerol) were used in 6-μL hanging drops at room temperature. Two yellow crystals large enough for X-ray diffraction experiments were obtained in one of the drops containing 0.2 M sodium acetate and 30% (w/v) PEG 4000 in 0.1 M Tris-HCl, pH 8.5. Upon optimization, however, we found that the use of PEG 6000 gave a more reproducible crystallization procedure with one or two large, high-quality crystals being obtained in each crystallization tray (i.e., per 24 drops). Figure 3 shows some of the best crystals obtained so far.

The crystals diffracted well in the X-ray beam and, for some larger crystals, diffraction spots were visible beyond 2 Å. Most crystals also showed little sign of radiation damage during data collection. A single crystal of approximate size 0.5 × 0.5 × 0.5 mm^3 was used to collect a native data set to 2 Å resolution. The 179,624 measurements were averaged to give 50,757 unique reflections with a completeness of 99.3% to 2.0 Å. The data collection statistics are given in Table 2. The crystals belong to the monoclinic system, space group $P2_1$, the $0k0$ reflections where k was odd, being systematically absent. The unit cell dimensions are $a = 54.19$ Å, $b = 109.23$ Å, $c = 67.17$ Å, and $\beta = 104.5°$. Given a protein molecular weight of about 34 kDa and two monomers in the asymmetric unit, the Matthews coefficient V_m

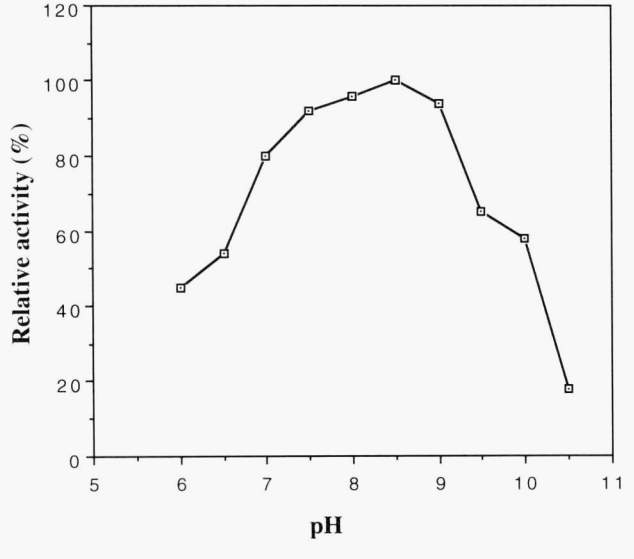

Fig. 2. pH optimum for dihydroorotate dehydrogenase A. Assays were performed in a buffer consisting of 50 mM Tris and 50 mM NaH_2PO_4, adjusted to the indicated pH either by addition of hydrochloric acid or NaOH. Assays contained 50 μM DCIP and 50 μM dihydroorotate as substrates and were monitored by measuring the absorption at 295 nm arising from the formation of orotate.

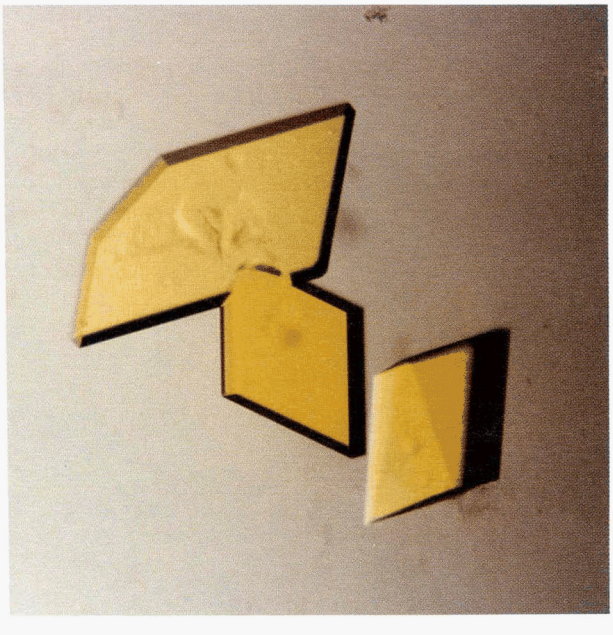

Fig. 3. Photograph of some of the best crystals obtained of dihydroorotate dehydrogenase A from *L. lactis*, the central crystal measuring about 0.4 × 0.4 × 0.2 mm^3.

Table 2. *A summary of the results from X-ray data collection and analysis*

	All data	Outermost shell
Resolution (Å)	25–2.0	2.03–2.00
No. of measurements	179,624	6,063
No. of unique reflections	50,757	2,337
Completeness (%)		
All data	99.3	90.8
$I/\sigma(I) > 2$ data only	85.7	56.3
$I/\sigma(I) > 3$ data only	80.3	45.4
Average $I/\sigma(I)$	22.4	4.1
R_{sym}[a] (%)	5.2	23.4

[a] $R_{sym} = (\Sigma|I - \langle I\rangle|)/\Sigma I$.

(Matthews, 1968) is 2.81. This gives a solvent content of about 56%.

A self-rotation function showed a peak significantly above the background, corresponding to a noncrystallographic twofold symmetry axis perpendicular to the crystallographic *b* and *c* axes. The existence of dihydroorotate dehydrogenase as a dimer with twofold symmetry is in accordance with the behavior of the enzyme in gel filtration experiments. The crystal packing of dihydroorotate dehydrogenase A thus conforms with the general picture that noncrystallographic symmetry elements most frequently are parallel or orthogonal to crystallographic reference directions (Wang & Janin, 1993).

Conclusions

The known sequences of the dihydroorotate dehydrogenases (from 17 species) seem to split into three main evolutionary families (not shown). It is our aim to study the structural basis for the functional differences between these three classes of dihydroorotate dehydrogenases, which appear to differ with respect to their preferences for electron acceptors and with respect to their subcellular localization. We hope that the high quality of the crystals described in the present paper will allow us to determine the three-dimensional structure of dihydroorotate dehydrogenase A from *L. lactis*, which could also pave the way for the analysis of members of the two other families of dihydroorotate dehydrogenases.

Materials and methods

Construction of an expression vector

The expression vector, pFN1, was constructed by cloning a PCR copy of the *pyrDa* gene from *L. lactis*, present on plasmid pKP9 (Andersen et al., 1994) into the multicopy plasmid pUHE23-2 (obtained from H. Bujard, Heidelberg), which carries the very strong LacI repressible $P_{A1/04/03}$ promoter to drive transcription of cloned genes. The PCR reaction was directed by two synthetic oligonucleotides (i.e., 5′-GCGGATCCGAGGAGTTT TTTAATGCTTAATACAACT and 5′-CCCAAGCTTGTTAT AATGATTTTAATTTTCC), which were designed to generate a *Bam*H I and a *Hin*d III site at the start and the end of the DNA fragment. The resulting PCR fragment and the vector pUHE23-2 were both digested with *Bam*H I and a *Hin*d III and, after removal of the 5′-phosphates from the digested vector, the two DNA fragments were ligated together by standard techniques. After transformation of the *E. coli* strain SØ6645 (*araD139 Δ(ara-leu)7679 galU galK Δ(lac)174 ΔpyrD(MluI-BssHII::Km*^{r})[F′ *proAB lacI*^{q}*ZΔM15* Tn*10*]) with the ligation mixture, pyrimidine prototrophic colonies that were resistant to ampicillin were selected on agar plates. Plasmids were isolated from 12 independent colonies and they all turned out to harbor plasmids similar to pFN1 (Fig. 1). The sequence of the cloned PCR fragment in pFN1 was determined by the technique of Sanger et al. (1977) using the Sequenase 2.0 kit (USB, Cleveland, Ohio) and found to be identical to the published sequence of the *pyrDa* gene of *L. lactis* (Andersen et al., 1994).

Purification of dihydroorotate dehydrogenase

Dihydroorotate dehydrogenase A was purified from strain SØ6645 carrying the expression vector pFN1 and grown to stationary phase at 37 °C with vigorous aeration in 8 L LB-broth (Miller, 1972) supplemented with 0.1 g ampicillin per liter. The synthesis of dihydroorotate dehydrogenase was induced by addition of 0.75 mM IPTG when the optical density (OD_{436}) of the culture was 0.8. Growth was continued for 24 h until the culture had been stationary for several hours. The cells were harvested by centrifugation for 20 min at 6,000 rpm using a GS3 rotor in a refrigerated Sorval centrifuge. The pellet was distinctly yellow.

The basal buffer used during all steps in the purification was 50 mM sodium phosphate, pH 6.0, containing 0.5 mM EDTA and 10% glycerol, termed Buffer A. The cells were suspended in 75 mL of ice cold Buffer A and disrupted by ultrasonic treatment using a Branson sonifier for 12 × 0.5 min, interrupted by cooling in an ice bath for 1.5 min between each cycle of sonication. Cell debris was removed by centrifugation as described above. Streptomycin sulphate (10%) was added to the yellow supernatant to a final concentration of 1%. The solution was stirred gently for 30 min at 4 °C and the precipitate, primarily consisting of nucleic acids, was removed by centrifugation for 30 min at 12,000 rpm in a refrigerated Sorval SS34 rotor. The extract was dialyzed for 1 h against 1 L of 5 mM sodium phosphate, pH 6.0, containing 10% glycerol and applied to an 80-mL column of DE52 cellulose (Whatman). After application of the sample, the column was first washed with 250 mL of Buffer A and then eluted with 500 mL of a linear gradient of 0–250 mM NaCl in Buffer A. The flow rate was 1 mL/min and 5-mL fractions were collected. The enzyme eluted from the column with the peak at approximately 0.2 M NaCl. The active fractions (200 mL) were pooled and dialyzed against 1 L of 5-mM sodium phosphate, pH 6.0, containing 10% glycerol for 3 h and the chromatography on the DE52 column was repeated. The active fractions from the second elution were pooled and applied to an 80-mL column of hydroxylapatite (Bio-Gel from Bio-Rad). After washing with 250 mL of Buffer A, the column was eluted with Buffer A containing 1 M NaCl. The fractions with most of the activity were pooled, concentrated to 5 mL using the Micro Ultrafiltration system from Amicon, and dialyzed exhaustively against Buffer A. For prolonged storage, glycerol was

added to a final concentration of 50% and the enzyme was kept at −20 °C.

Assays of dihydroorotate dehydrogenase activity

In the standard assay for dihydroorotate dehydrogenase activity, the oxidation of dihydroorotate was coupled to the reduction of the synthetic quinone DCIP. The reduction of 1 μmol DCIP causes a decrease in the absorbance at 600 nm, $\epsilon = 20 \times 10^3\ M^{-1}\ cm^{-1}$ (Karibian, 1978). The spectra were recorded in a Zeiss Specord S10 diode-array photometer. The standard assay mixture contained 0.1 M potassium phosphate, pH 7.0, 5 mM KCN, 0.1% Triton X-100, and 50 μM DCIP. The assay temperature was 37 °C. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol orotate per min under these conditions. In other assays, using different electron acceptors, we used the absorption at 295 nm to obtain a quantitative measure of the production of orotate: ($\epsilon = 3.67 \times 10^3\ M^{-1}\ cm^{-1}$).

Determination of the flavin cofactor

The flavin was released from an aliquot of the enzyme by treating with 0.25 M formic acid and analyzed by chromatography on ion exchange thin-layer plates together with authentic FMN and FAD as described by Larsen and Jensen (1985). In addition, a 1.0 mg/mL solution of the enzyme was denatured by heating at 80 °C for 20 min. After clearing of the solution by centrifugation, the spectra were recorded on a Specord S10 (Zeiss) and compared with the spectra of solutions of authentic FMN and FAD.

X-ray diffraction analysis

The diffraction data were collected at 15 °C with an R-axis II imaging plate system. X-rays were generated with a Rigaku Rotaflex RU200 rotating copper anode operating at 50 kV and 180 mA using a graphite monochromator and a 0.5-mm collimator. Seventy-two diffraction images were recorded, each with an oscillation range of 2.5° and an exposure time of 30 min, giving a total rotation range of 180°. These images were processed using the programs DENZO and SCALEPACK (Otwinowski, 1993).

The self-rotation function was calculated in steps of 1° using the program AMORE (Navaza, 1994) for data in the resolution range 20–6 Å and an integration sphere of radius 15 Å.

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